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Antimicrobial activity of marine bacteria isolated from Gulf of Mexico

Actividad antimicrobiana de bacterias marinas aisladas del Golfo de México

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Abstract

Currently there is a need for new antibiotics with an alternative mode of action and new chemical structures. Bacterial pathogens are gradually becoming more resistant to conventional antibiotics, generating an emergence of infectious diseases and they are becoming a great problem in the field of public health. In this study, seven different isolated bacteria were obtained from offshore seawater and sediment of the Gulf of Mexico from Campeche, Mexico. They were substance producers which inhibit growth of human pathogens like *Staphylococcus aureus* and *Pseudomonas aeruginosa* and one of them was a polymer producer on peptone and glucose culture. They were characterized phenotipically by means of morphological techniques and physiologically by conventional tests. Four of them were Gram-positive bacteria and the Scanning Electron Microscope analysis revealed their size between 0.6 – 1.5 µm. One of seven marine strains, Gram negative, yellow pigmented, slightly curved rods, was identified as *Pseudoalteromonas* sp. on the analysis of the gen16S rRNA sequence.

Keywords: marine bacteria, marine antibiotic, *Pseudoalteromonas*, resistant pathogens.

Resumen

Hoy en día existe la necesidad de encontrar antibióticos con nuevas estructuras químicas y modos de acción alternativos. Se ha observado que bacterias patógenas comunes progresivamente desarrollan resistencia al tratamiento con antibióticos tradicionales, surgiendo y resurgiendo enfermedades infecciosas que generan un gran problema en salud pública. En este estudio, se obtuvieron siete colonias bacterianas pigmentadas de agua de mar y de sedimento marino procedente de las costas de Campeche, México. Las colonias aisladas produjeron sustancias que inhibieron el crecimiento de bacterias patógenas a humanos como *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Las bacterias marinas fueron caracterizadas fenotípicamente de acuerdo a su morfología microscópica y por pruebas fisiológicas convencionales. Cuatro de los aislados resultaron ser bacterias Gram positivas y las otras tres fueron Gram negativas. Cuando se observaron por microscopía electrónica de barrido, su tamaño aproximado fue entre 0,6 – 1,5 µm. Uno de los aislados fue una colonia amarilla con bacilos cortos Gram negativos y ligeramente curvos, identificado por la secuencia del gen16S rRNA como *Pseudoalteromonas* sp.

Palabras clave: bacteria marina, antibiótico, *Pseudoalteromonas*, patógenos resistentes.

Introduction

Currently emerging and reemerging infectious diseases are a major problem in public health and global economies. They are caused by different types of infections such as drug – resistant infections, mostly involving bacteria, and many emerging pathogens are increasing significantly over time (Jones et al. 2008) because they are becoming progressively more resistant to conventional antibiotic compounds. For example, *Pseudomonas aeruginosa* has been reported as an opportunistic pathogen and one of the most common causes of nosocomial infections by the intrinsic resistance to many antimicrobial agents (Kollef 2005, Depardieu et al. 2007). In *Staphylococcus aureus*, virulence and antibiotic resistance are contributing to its emergence as leading overall cause of nosocomial infections in both nosocomial and outside the hospital setting (Lowy 2003, Livermore 2004).

Therefore, there is a need for a great variety of substances with antibiotic activity, a different mode of action and chemical structures. On the other hand, the chemical drugs synthesis has been a good option; however the diversity of microorganisms and their secondary metabolites is unrivaled and unmatched in medical significance (Fenical 1993). Microorganisms are a prolific source of structurally diverse bioactive metabolites and have yielded some of the most important products of the pharmaceutical modern industry (Cragg & Newman 2001).

Although marine organisms do not have a significant history of use in traditional medicine, in the last years marine microorganisms have become an important point of study in search of novel microbial products showing antimicrobial activities, antiviral, immunosuppressives, enzyme inhibitor metabolites, receptor antagonists, antitumor activities and anticoagulant properties (Carte 1993, Jensen & Fenical 1994, Reichenbach 2001). Marine natural products contain a wide range of novel antibiotics with unique complex structures not present in the terrestrial environment. The isolation of highly brominated compounds illustrates that marine bacterias have common mechanisms to incorporate bromine or other halogens into organic compounds that can potentially lead to enhance bioactivities (Bernan et al. 1997).

Until recently, microbiologists were greatly limited in their studies of natural microbial ecosystems, because of disability to cultivate microorganisms out of their environment. Today, the bacterial growth from marine sediment takes place and it is well known (Kaeberlein et al. 2002) and procedures based in the extraction of nucleic acid from environmental samples have permitted the identification of microorganisms through the isolation and sequencing of ribosomal RNA o rDNA (Handelsman et al. 1998). Therefore, the recent development of procedures for cultivation and identifying microorganisms has aided microbiologists in their assessment of the earth's full range of microbial diversity (Munn 2004).

Their metabolic and physiological capacities allow microorganisms to survive virtually across all environmental conditions found in the Earth. The adaptation of bacteria to diverse marine

habitat can determine the special skills for the production of unique secondary metabolites (Bernan et al. 1997). The microorganisms can even show properties different from terrestrial known species. For example the Gram positive marine halophilic bacteria *Actinopolyspora* AH1, isolated from the west coast of India, showed resistance or tolerance to some antibiotics and good antibacterial activities against Gram positive bacteria (Kokare et al. 2004).

In this work, seven strains were isolated from offshore seawater and sediment of the Gulf of Mexico from Campeche. They were substance-producers which inhibit growth of drug-resistant human pathogens like *Staphylococcus aureus* and *Pseudomonas aeruginosa* species.

Material and methods

Isolation and culturing of the microorganisms

The microorganisms were obtained of seawater and sediment samples from the Gulf of Mexico, Campeche. Samples were collected 10 m of depth and 32 Km offshore, in November of 2007. The salinity was 3,8% and the pH 8.0. The sediment and water samples were collected in sterile 50 mL conical tubes. Samples were kept cool during the expedition and processed as soon as possible until our return to the laboratory.

Approximately 50 mg of wet sediment or 1 mL of seawater sample were used to inoculate agar plates supplemented with 2 $g.L^{-1}$ sodium caseinate, 0.1 $g.L^{-1}$ asparagine, 0.001 $g.L^{-1}$ ferrous sulfate and 0.075 $g.L^{-1}$ ferric citrate dissolved in natural seawater. The final pH of the medium was adjusted to 8.0 before sterilization. Agar medium was prepared with 100% natural seawater sterilized by filtration. The inoculated plates were incubated at 28 °C for three weeks. After isolation, the colonies were purified by streak plate method and kept in 30% glycerol diluted in seawater-medium at –70 °C and recultured as required.

Culture conditions.- The isolated colonies were cultured for 72 h in 50 mL of YPG medium, consisting of: 4 $g.L^{-1}$ yeast extract; 5 $g.L^{-1}$ casein peptone and 10 $g.L^{-1}$ glucose; 2 $g.L^{-1}$ $NaNO_3$; 0.01 $g.L^{-1}$ $FeSO_4$; 0.5 $g.L^{-1}$ $K2HPO_4$; 0.5 $g.L^{-1}$ $MgSO_4$ on filtered natural seawater and adjusted pH 8.0. These cultures were shaken at 150 rpm and 28 °C. Starter cultures were generated by inoculated 0.5 mL of subcultures from frozen culture (medium plus 30% glycerol) and grown under the same conditions. The effect of the composition of the kind of water used for cultivation was determined using YPG medium dissolved in natural seawater, artificial seawater or distiller water.

Crude extract.- Marine bacterial cells were separated from the spent broth by centrifugation (at 3000 *g* for 15 min at 4 °C) and washed twice with sterile natural seawater. Two grams of biomass were resuspended in 10 mL of PBS and sonicated. Spent broth and crude extract was tested for antimicrobial activity.

Determination of antimicrobial activity

Antibacterial activity was tested against clinical pathogen which included: Gram positive bacteria *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* S clinical isolate sensible to ampicillin (AM), ceftazidime (CAZ), cefataxime (CXM), gentamicin (GM) and *Staphylococcus aureus* R MRSA clinical isolate resistant to AM, GM, CAZ and Gram negative bacteria such as *Pseudomonas aeruginosa* R clinical isolate resistant to cefataxime (CTX),amikacin (AN).

Paper disks impregnated with 20 µL of crude extracts or spent broth free of cells were used for antimicrobial activity assay using the conventional diffusion plate method. Inhibition zones around the disk indicated antibacterial activity which was measured after 24 h of incubation. Different antibiotics (ampicillin (AM-10), ceftazidime (CAZ-30), vancomycin (VA-30), amikacin (AN-30), cefataxime (CTX-30), imipenem (IPM-10), cefurox (CXM-30), sulfamethoxazole with trimethoprim (SXT), gentamicin (GM-120) were used as positive or negative controls in the plates. For antibiotic sensitivity the isolated (MS-3/48) was tested on YPG agar in natural seawater plates and antibiotics IPM-10, CTX-30, AN-30, CF-30 from commercial BD BBL™ Sensi-Disks™ Antimicrobial Susceptibility Test Discs were used. The determinations were performed at least thrice and the averages of the values are reported.

Pigment extraction in acetone-methanol from strain (MS-3/48).- We selected the isolated (MS-3/48) and it was harvested by centrifugation. Yellow pigment was extracted from the cells with acetone-methanol (7:2 volume in volume) at 4 °C for 12 h in dark (Du et al. 2006). After that the extract was evaporated off in vacuum below 30 °C. The concentrate extract was tested for antimicrobial activity bioassay and methanol (5%) was also used as a negative control.

Determination of cell dry weight.- A 25 mL amount of culture marine bacteria was filtered throughout 0.45 µm membrane (millipore) under vacuum, the mass retained on the filter was washed with 25 mL of sterile natural seawater. The washed cells were dried in a microwave oven at constant weight and weighed. Every determination was performed at least twice and the averages of the values are reported.

Biochemical test.- Additional biochemical tests were carried out on one of seven isolates (MS-3/48) using API 20NE and API50 CH test kit (bioMérieux) as described by the manufacturer, with the exception that the strain was suspended in sterile natural seawater.

PCR amplification of the 16S RNA gene

Amplification of the 16S ribosomal DNA (rDNA) sequence of strain MS-3/48 was carried out. First, 10 to 20 ng of purified genomic DNA was amplified in 50 µL of a reaction mixture consisting of 20 mM Tris-HCl (pH 8.4), 1,5 mM $MgCl_2$, 200 µM of each dNTP and 2 U of *Taq* DNA polymerase. The PCR primers used for amplification forward(5' –CTYAAAKRAATTGRCGGRRRSSC- 3' , *E. coli* positions 909-932) and reverse (5' – CGGGCGGTGTGTRCAARRSSC - 3' , *E. coli* positions 1383 – 1404) at a final concentration of 0.2 µM as was described by Rivas (2004). PCR conditions were as follows: pre-heating at 95 °C for 5 min. The thermal profile consisted of 35 cycles of denaturing at 95 °C for 1 min; this was followed by an annealing step at 55 °C for 2 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min.

The amplified product was checked by gel electrophoresis and a 400 bp DNA band was excised and purified using the DNA extraction kit Quiagen. The sequence reaction was performed on a Perkin-Elmer Genetic Analyzer 310. The primers used were the same employed in the PCR amplification. The sequence obtained was compared against those in data bank using a BLAST program.

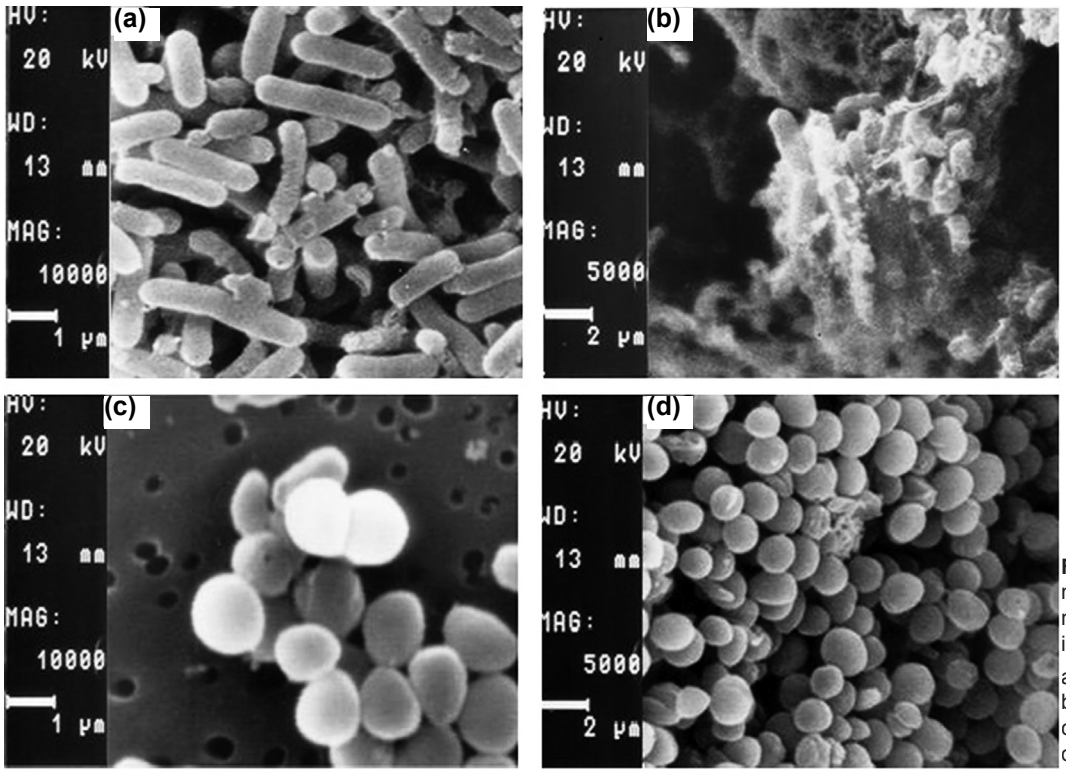

Figure 1. Scanning electron micrographs showing general morphology of marine isolates.
a) strain MS-1/48 x 10,000,
b) strain MS-3/48 x 5,000,
c) strain MS-6/48 x 10,000,
d) strain MS-7/48 x 5,000.

Scanning electron microscopy

For scanning electron microscope, four isolates (named MS-1/48, MS-3/48, MS-6/48 and MS-7/48) were fixed at 4 °C for 1 h in 2.5% glutaraldehyde diluted in Millipore- filtered sea water, washed in the same water, post-fixed at 4 °C for 30 min in 1% OSO_4 in sea water. The sample was filtered through a 0.22-µ-pore-size filter. The filters were serially dehydrated with increasing ethanol concentrations (twice for 10 min in each stage) and dried at critical-point. The samples were mounted on scanning electron micrograph stubs, splatter coated with gold and viewed on a Zeeis DSM- 950 scanning electron microscope.

Results

Under isolation method evident growth of marine bacteria appeared on seawater agar plates after three weeks of incubation. During the course of screening for antimicrobial activity, 400 marine strains were isolate and 48 (12%) showed to be pigmented strain. Seven (14.5%) out of 48 pigmented strains reveled antimicrobial activity against test bacteria as consequence only them were selected and named (MS-1/48, MS-2/48, MS-3/48, MS-4/48, MS-5/48, MS-6/48 and MS-7/48). Table 1 shows the antimicrobial activities from marine isolates bacteria. They showed to be substance-producers which inhibited growth of the bacteria pathogens *S. aureus* and *P. aeruginosa*. The marine strains, (MS-1/48), (MS-2/48), (MS-5/48) and (MS-6/48), showed a marked activity against Gram positive *S aureus* bacteria, but isolates (MS-3/48), (MS-4/48) and (MS-7/48) besides showed activity against Gram negative *P. aeruginosa,* although none of seven strains revealed activity against *E. coli*.

They were pigmented colonies and the strains (MS-1/48), (MS-2/48), (MS-6/48) and (MS-7/48) were Gram positive cells and the strains (MS-3/48), (MS-4/48) and (MS-5/48) were Gram negative cells (Table 2).

When marine bacteria were grown in liquid YPG medium they exposed good growth after 48 h in presence of natural seawater or artificial seawater but non in YPG dissolved in distiller water.

Observations on light microscopy revealed that strain MS-2/48 was rod-shaped and the strains (MS-4/48) and (MS-5/48) were coccus- shaped cells. Scanning electron microscopy of isolated (MS-1/48), (MS-3/48), (MS-6/48) and (MS-7/48) showed the general morphology of cells and size (Fig. 1). Cells from strains (MS-1/48) (1a) and (MS-3/48) (1b) confirmed to

Table 1. Antimicrobial activity against reference human pathogen strains.

Isolated marine strains	Zone of inhibition (mm)[a]					
	S. aureus ATCC 25923	*S. aureus* S[b]	*S. aureus*[c] MRSA	*E. coli* ATCC 25922	*P. aeruginosa* S[d]	*P. aeruginosa* R[e]
MS-1/48	0	9	9	0	0	0
MS-2/48	0	10	12	0	0	0
MS-3/48	15	14	19	0	10	10
MS-4/48	12	13	13	0	9	8
MS-5/48	13	16	16	0	9	0
MS-6/48	20	17	16	0	9	0
MS-7/48	14	16	16	0	13	13

[a]Diameter of inhibitions zone; [b]Clinical isolated sensible to AM, CAZ,CXM, GM; [c]Clinical isolated resistant to AM, GM, CAZ; [d]Clinical isolated sensible to CAZ; [e]Clinical isolated resistant to CTX, AN

Table 2. General morphology characteristics of isolated marine strains.

Isolated marine strains	Pigment of strain	Gram stained	Morphology	Sized (µm) SEM
MS-1/48	Violet	(+)	Rod-shaped	1,08 – 1,48
MS-2/48	Red	(+)	Rod-shaped	ND
MS-3/48	Yellow	(-)	Rod-shaped	0,6 – 1,5
MS-4/48	Pink	(-)	Coccus	ND
MS-5/48	Pink	(-)	Coccus	ND
MS-6/48	Pink	(+)	Coccus	0,83 – 1,48
MS-7/48	Pink	(+)	Coccus	0,65 – 1,0

ND not determined

be rod-shaped while cells strains (MS-6/48) (1c) and (MS-7/48) (1d) were coccus- shaped bacteria and ranged 0.65 to 1.5 µm in size. Besides, when isolated (MS-3/48) grew on glucose and peptone medium was polymer- producer and it was observed with SEM to bacterium encase in extracellular matrix material as shown in Figure 1b, similar to microorganims that grow in bofilms.

Data of antimicrobial capacity against target bacteria were the criteria to select isolated (MS-3/48) for molecular analyses and API20 NE, API50 CH test.

The API20 NE test exhibited strain (MS-3/48) to be nitrate reductase negative, oxidase positive, citrate assimilation positive but weak glucose oxidation (Table 3). From 50 substrates and derivates only weak D-glucose utilization was observed in the API50 CH test (data not shown) and showed to be resistant to imipenem (IPM), cefalotina (CEP), amikacine (AMK) tests.

By separating the bacterial cells from the spent broth and the yellow pigment from the cells we were able to investigate the intracellular or extracellular antimicrobial activity. Crude extracts were prepared from sonicated biomass and then antimicrobial activity assay was performed indicating that antibiotic capacity resided in biomass but not in spent broth free of cells or in the pigment of cells (data not shown).

Table 3. Conventional test (API 20NE) for MS-3/48 Gram negative.

NO_3	(-)
TRP	(-)
GLU	(+)
ADH	(-)
URE	(-)
ESC	(+)
GEL	(+)
PNG	(-)
OX	(+)
GLU A	(+)
CIT A	(+++)

(-) not growth; (+) weakly growth; (+++) good growth

NO_3, potasic nitrate; TRP, L-tryptophane; GLU, D-glucose oxidation; ADH,L-arginine; URE, Urea; ESC, ferric citrate esculin; GEL, gelatin; PNG, 4-nitrophenil-D-galactopiranoside; OX, oxidase; GLU A, glucose assimilation; CIT A, citrate assimilation.

In order to approach the genus to which the isolated cells could belong, we carried out 16S rRNA gene - PCR based method. When the BLAST was performed the sequence of the marine bacteria showed to be a part of bacterial species most closely related to *Pseudoalteromonas* sp. revealed 97% of similarity (Fig. 2).

Discussion

Marine bacteria showing antibacterial activities have been described for more than 50 years (Rosenfeld & Zobell 1947). However, at present, the literature in México about microbial diversity of our coast is disperse or scarce particularly in relation to microorganisms producers of antimicrobial agents against

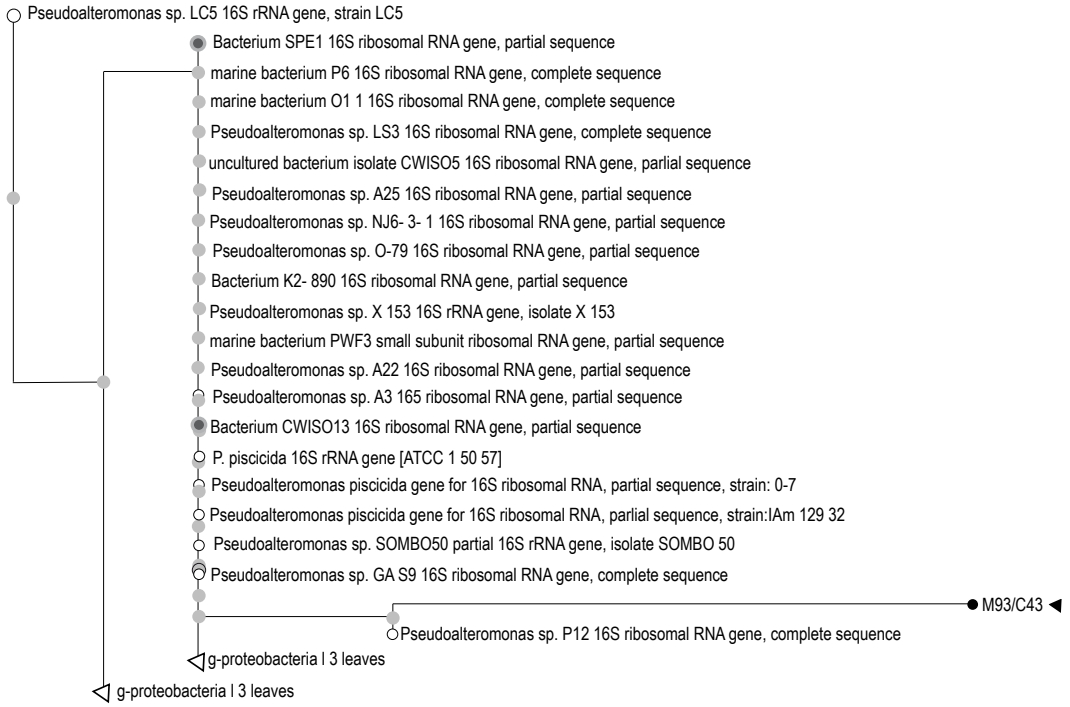

Figure 2. Dendogram of the relatedness of strain MS-3/48 with several *Pseudoalteromonas* species based on the 16S rDNA sequences. The Blast tree was constructed by neighbor joining analysis.

human bacterial pathogens. In this study we have isolated and selected seven strains from offshore seawater and sediment in Campeche, México. They were Gram positive and Gram negative bacteria and showed antimicrobial activity against two bacteria reported as leading pathogen *S. aureus* and *P. aeruginosa* (Table 1). Therefore, these antimicrobial marine agents could be an important therapeutic alternative particularly those directed against multiresistant Gram negative or Gram positive bacteria in hospitals. On the one hand, infections due to *S. aureus* are highly prevalent in pediatric patients and healing options are limited (Isnansetyo & Kamei 2003, Depardieu et al. 2007). On the other hand, Gram negative *P. aeruginosa* has been reported as one of the most common causes of nosocomial infections by the intrinsic resistance to almost all available antimicrobial agents (Livermore 2004, Kollef 2005, Depardieu et al. 2007).

During antimicrobial spectrum determination from marine bacteria it was perceived that the Gram negative human bacterial pathogens were not as susceptible to marine antagonists as were the Gram positive human bacterial pathogens; noted by inhibition zone against *P. aeruginosa* or *E. coli,* it was smaller than obtained against *S. aureus* as is shown in Table 1, a fact noted in accordance with the general observations made in marine microorganisms (Rosenfeld & ZoBell 1947, Saadoun et al. 1999, Basilio et al. 2003).

Besides they confirmed their marine nature because the marine isolated did not expose growth in the YPG medium dissolved in distilled water which indicated the dependence on seawater.

In this report, the seven isolates pigmented were bioactive compounds producer strains (Table 2). In accordance with this, it is a recognized fact that a likely association exists between pigments and toxic activity in several marine pigmented heterotrophic bacteria; for example, a number of biosynthetic enzymes involved in synthesis of inhibitors compounds were identified for pigment synthesis in *Pseudoalteromonas tunicata* (Lichstein & van de Sand 1945, Holmström et al. 1996, Egan et al. 2002). However, when we tested yellow pigment of strain (MS-3/48) against target pathogen bacteria, no antimicrobial activity was observed indicating that pigment was not the antimicrobial compound against target bacteria.

During observation with light and electron microscopes these marine bacteria were found to be coccus and rod-shaped cells and ranged from 0.65 to 1.5 µm of size (Figs. 1 a, b, c, d) according to dimensions of the representative marine prokaryotes (Munn 2004).

On the other hand, some marine bacteria can also produce compounds as exopolymers to provide a means by which bacteria can adhere to surfaces and grow in biofilm (Holström & Kjelleberg 1999). We report a rod-shaped bacterium (strain MS-3/48) which was also producer of exopolymer as is shown in Fig. 1b. Our results may suggest that isolated MS-3/48 is biofilm-forming bacterium and its extracellular polymeric compound helps the cell to avoid nutrient- depleted environments, which could enhance the chances for other marine organisms to survive in specific marine habitats as it was described for marine bacteria genus *Pseudoalteromonas* by Bowman (2007).

The crude extracts from biomass showed antibiotic activities against *S. aureus* and *P. aeruginosa* but non in pigment extracted from the cells or in the spent broth free of cells suggested that biological compounds with antimicrobial capacity were into the cells or it may be adhered to extracellular polymeric matrix close to the cells as describe in a marine *Chromobacterium* by Andersen et al. (1974).

On the other hand, phenotypic analysis showed that the cells from isolated MS-3/48 were Gram negative bacteria, aerobic, rod shaped cells, 0.65 - 1 µm long, yellow pigmented cells, oxidase positive and nitrate reductase negative; sodium ions are essential for the growth of these cells. Moreover, it was characterized by hydrolisis of gelatine, citrate assimilation positive but weak glucose oxidation (Table 3) and it was resistant to imipenem (IPM), cefalotina (CEP), amikacine (AMK) test. In addition, the isolates (MS-3/48) observed some characteristics of marine bacteria genus *Pseudoalteromona* as gelatinase activity, weakly utilization of D-glucose and positive for oxidase as was reported by Ivanova et al. (2004) and Bowman, (2007). The 16S RNA gene sequence analysis and Blast queries against the latest release of the bacteria division of GeneBank ranged from 97% among *Pseudoalteromonas* sp. genus. In conclusion, mexican offshore may offer an opportunity of biological resource for antimicrobial therapeutic agents.

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