Archives of Microbiology
https://doi.org/10.1007/s00203-020-01851-w

ORIGINAL PAPER

Marine bacteria associated with shallow hydrothermal systems in the Gulf of California with the capacity to produce biofilm inhibiting compounds

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Received: 20 August 2019 / Revised: 9 December 2019 / Accepted: 1 March 2020

Abstract
Shallow hydrothermal systems are extreme environments. The sediments and fluids emitted from the vents present unusual physical and chemical conditions compared to other marine areas, which promotes unique biodiversity that has been of great interest for biotechnology for some years. In this work, a bioprospective study was carried out to evaluate the capacity of bacteria associated with shallow hydrothermal vents to produce biofilm-inhibiting compounds. Degradation assays of *N*-acyl homoserine lactone (AHL) autoinducers (C6HSL) involved in the quorum sensing process were carried out on 161 strains of bacteria isolated from three shallow hydrothermal systems located in Baja California Sur (BCS), Mexico. The biosensor *Chromobacterium violaceum* CV026 was used. Twenty-three strains showed activity, and organic extracts were obtained with ethyl acetate. The potential of the extracts to inhibit the formation of biofilms was tested against two human pathogenic strains (*Pseudomonas aeruginosa* PAO1 and *Aeromonas caviae* ScH3), a shrimp pathogen (*Vibrio parahaemolyticus* M8), and two marine strains identified as producing biofilms on submerged surfaces (*Virgibacillus* sp C29 and *Vibrio alginolyticus* C96). The results showed that *Vibrio alginolyticus* and *Brevibacillus thermoruber,* as well as some thermotolerant strains (mostly *Bacillus*), produce compounds that inhibit bacterial biofilms (*B. licheniformis, B. paralicheniformis, B. firmus, B. oceanizedimenis, B. aerius and B. sonorensis*).

Keywords Quorum sensing · Bacillus · Pathogen · Biofilm · Extracts

Introduction

The study of microorganisms that inhabit extreme environments is of particular interest. They are not only able to survive but can also actively grow and multiply at high temperatures and under chemical conditions not found in other environments, such as high concentrations of carbon dioxide, hydrogen sulfide, hydrocarbons, heavy metals, and other conditions that make colonization of other organisms impossible (Sonnleitner 2005; Lentini et al. 2014).

The microorganisms that inhabit hydrothermal systems have generally been studied with an ecological approach to elucidate the function they have in the environment (Van Dover 2000). Have been found bacteria that can colonize diverse substrates and transform chemical compounds derived from hydrothermal activity through redox reactions and chemosynthetic C-fixation (Fisher et al. 2007; Jogersen and Boetius 2007).

This process promotes the formation of hydrothermal systems with its own biodiversity, which for some years have been of great interest for studies of biotechnological potential because some bacteria isolated from these environments are producers of autoinductors involved in the formation of exopolymers. These compounds, genetically expressed for the formation of biofilms, allow them to survive in adverse environmental conditions because of bacterial communication at high cell densities (quorum sensing) (Nichols et al. 2009). *N*-acyl homoserine lactonase-producing thermophilic bacteria have been found (Seo et al. 2011; Morohoshi et al. 2015), as well as compounds that degrade the autoinductors of other bacteria. This degradation inhibits communication and formation of biofilms, which is interesting for

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Published online: 20 March 2020

applications in the area of biomedicine and biotechnology (Kokare et al. 2009).

The process of quorum sensing (QS) allows coordination of microbial behaviors in mesophilic bacteria communities. Little research has been done to determine if QS occurs in extreme environments. Initially, the QS process was thought unlikely in thermophilic and moderately thermophilic environments because signaling molecules of the AHL type are thermolabile (Nichols et al. 2009). Despite this, some studies show that thermophilic and hyperthermophilic microorganisms communicate with different purposes and some of them have been identified as exopolysaccharide producers, as well as biofilm formers. In addition, it has been found that in hydrothermal environments, not only the LuxS gene can mediate AI2 production but also temperature (Johnson et al. 2005; Nichols et al. 2009).

The presence of *N*-acyl homoserine lactonase enzymes in thermophilic bacteria has been demonstrated and has AHL-degrading activity of different lengths of acyl chain (Seo et al. 2011; Morohoshi et al. 2015). The presence of a lactone enzyme similar to a phosphotransferase has also been demonstrated in the thermophilic archaea *Sulfolobus solfataricus.* It has been shown to have an inhibitory activity of QS against *Pseudomonas aeruginosa* PAO1, providing the first demonstration that these enzymes can be used to attenuate the production of factors of virulence controlled by QS signals (Ng et al. 2011).

The communication between bacteria through the QS unchaining the synchronized expression of genes in the population, regulating important biological functions, such as mobility, bioluminescence, virulence, or the formation of biofilms (Dobretsov et al. 2009; Ng et al. 2011; Kothari et al. 2017), in relation to the latter, is produced to protect against drying out. It is also known that bacteria immersed in their biofilms are more virulent and have greater resistance to current antibiotics (Costerton et al. 1999; Davis 2003).

Some opportunistic pathogens, such as *Serratia marcescens* and *Pseudomonas aeruginosa* regulate the production of their virulence factors and the formation of biofilms by means of QS systems. It is known that in the genome of *P. aeruginosa,* more than 6% of the genes are regulated by QS and are involved in the control of pathogenesis (Schuster et al. 2013; Wagner et al. 2003). Several studies have been carried out in which the objective has been to control the microbial pathogenesis by inhibiting the formation of QS or biofilms. This seems to be an attractive alternative because it is less likely to develop bacterial resistance (Sommer et al. 2013).

There are numerous investigations studying how to prevent communication between bacteria and to inhibit, or delay, the process of biofilm formation. However, to date, all the "messenger molecules" involved in this process are unknown. For this reason, the identification of compounds that interfere with QS systems is of considerable importance for the development of treatments against pathogens associated with biofilms (Christersen et al. 2007).

The marine environment contains great microbial diversity, and the microorganisms present are potential producers of bioactive compounds. As such, it represents an important resource for the search for new inhibitory compounds of the QS (Dobretsov et al. 2009; Teasdale et al. 2011; Yaniv et al. 2017). It should be noted that most of the studies published on the production of QS inhibitors by marine bacteria have focused on bacteria isolated from surfaces, biofilms, and sediments (Teasdale et al. 2009, 2011). Many of the known anti-QS compounds have been found in sessile marine organisms, such as sponges and microalgae, which interact closely with bacteria (Stowe et al. 2011).

Therefore, the bases must first be laid on which bacteria have the potential to inhibit such communication for future biotechnological applications, in this case, marine bacteria associated with shallow hydrothermal systems with potential production of effective compounds.

Materials and methods

Study area and sample collection

The samples were collected in three shallow hydrothermal systems found in Baja California Sur, Mexico: Mapachitos (26° 40′ 27.58 ″N, 111° 50′ 37.13″ W), mangrove of Santispac (26° 45′ 46.21″ N, 111° 53 ′36.91″ W), and Sargento beach (24° 6′ 51.01 ″N, 109° 59 ′54.80″ W).

Samples of water and sediment were taken from the cracks observed or where the highest temperatures were recorded (in case of not observing cracks in the sediment) either by SCUBA diving or at the coastline.

Isolation and bacterial characterization

Samples were serially diluted and transferred (10^5) to Marine agar (MA). The plates were incubated at 35 °C and 60 °C for 24 to 96 h, and the colonies developed were isolated. The bacterial colonies were purified by the streak plate method on MA. In order to know if the strains were thermotolerant or thermophilic, they were inoculated in MA in duplicate, with incubation temperatures of 35 and 60 °C.

Evaluation of the presence of quorum sensing inhibitory activity (IQS)

In this assay, the mutant biosensor strain *Chromobacterium violaceum* (CV026) was used, which easily detects a wide range of AHL signaling molecules (McClean et al. 1997). To

detect the presence of IQS activity of the isolated strains, we followed the method outlined by Romero and Otero (2010) with some modifications. The strains were inoculated in 1.5 mL microtubes with marine broth (MB) under their respective incubation conditions (35 °C or 60 °C). After 24 h, 40 µL of C6HSL signaling molecules (*N*-hexanoyl-L-Homoserine lactone) previously diluted in DMSO was added. The tubes remained at room temperature for 24 h and centrifuged at 10,000 rpm for 15 min. At the same time, the strain *C. violaceum* CV026 was inoculated at an optical density of 1 (585 nm) in LB-Kan liquid medium (LB medium + 25 µg/mL kanamycin) and streaked in plates with LB agar. The plates were perforated with sterile punches. In the wells, 40 µL of the supernatant of the tubes with the strains and signaling molecules were added. As a positive control, LB medium was used, and as a negative control, LB medium with the same amount of signaling molecules was used. Finally, the plates were incubated at 30 °C for 24 h. This test was performed in triplicate.

Extraction of compounds from bacterial cultures

The active strains were inoculated in MB and incubated 96 h at 35 and 60 °C, depending on their requirements. The cultures were centrifuged at 10,000 rpm for 15 min; the supernatants were obtained; and liquid–liquid extractions were performed. The supernatant was emptied into a separatory funnel. Ethyl acetate (1 L ethyl acetate: 100 µL glacial acetic acid) was added and stirred vigorously. The extracts were evaporated to dryness at 45 °C under reduced pressure. This procedure was performed three times (Lule 2007; Nithyanand and Pandian 2009). The extract was preserved by freezing until used in subsequent tests.

Bacterial biofilm inhibition assays

The activity of organic extracts as inhibitors of biofilm formation was tested using the following biofilm-forming bacteria: *Virgibacillus* sp. C29 and *Vibrio alginolyticus* C96, which were chosen for their involvement in biofouling of submerged marine surfaces; the two human pathogens *Pseudomonas aeruginosa* PAO1 and *Aeromonas caviae* ScH3; and the marine bacterium *Vibrio parahaemolyticus* M8, associated with both human and shrimp infections in farming farms. The strains were inoculated in their respective media and incubated for 24 h (the two strains of biofouling and *V. parahaemolyticus* M8 in MB at 35 °C, *P. aeruginosa* PAO1 in LB at 37 °C, and *A. caviae* in soybean triptysein (TSB) at 30 °C). Three dilutions of the extracts obtained with nuclease-free water were made at concentrations of 1000, 100, and 10 µg/mL.

The biofilm-forming strains were adjusted to an optical density of 1 (585 nm). One hundred µL of the culture was placed in 96-well microplates, and subsequently, the same amount of the different dilutions of the extracts was added. The test was performed in triplicate. The strain inoculated in the medium without extract was counted as a negative control, and the culture medium was blank. After incubating for 48 h, the broth with bacteria was removed from the plates. Three washes were carried out with sterile distilled water and allowed to dry. A 1% crystal violet (w/v) solution was added, and after 45 min it was removed. Three washes were performed with sterile distilled water, and 200 µL of 96° ethyl alcohol was added. The results were read in at 595 nm in a microplate reader (Álvarez et al. 2006).

Growth and biofilm formation kinetics

The target strains (*Virgibacillus* sp. C29, *Vibrio alginolyticus* C96, *Pseudomonas aeruginosa* PAO1, and *Aeromonas caviae* ScH3) were inoculated into 96-well microplates in their respective culture media, and their growth was evaluated at hours 0, 4, 8, 24, 30, and 48, by reading its optical density at 620 nm. Simultaneously, the methodology indicated in the previous section was repeated to evaluate the kinetics of biofilm formation and the effect of the extracts at 4, 8, 24, 30, and 48 h. The readings were made in a microplate reader at 595 nm.

Molecular identification of active bacteria

The phenol–chloroform method was used for DNA extraction (Sambrook & Russell 2001). Briefly, 24-h bacterial cultures were centrifuged at 14,000 rpm for 10 min, and the supernatant was discarded. The button was resuspended in 575 µL of TE pH 8.0, 30 µL of 10% SDS, and 3 µL of proteinase K (10 mg / mL). The solution was mixed and incubated for 2 h at 37 °C. Then 100 µL of 5 M NaCl and 80 µL of CTAB were mixed and incubated for 10 min at 65 °C. Finally, 0.8 mL of phenol:chloroform:isoamyl alcohol (25:24:1) was added, homogenized, and centrifuged for 10 min at 14,000 rpm. The aqueous phase was recovered and placed in a new tube. A volume of chloroform--isoamyl alcohol was added (24:1), mixed, and centrifuged 10 min at 14,000 rpm. The supernatant was recovered. Two volumes of isopropanol were added. The samples were preserved at − 20 °C for 24 h. Subsequently, they were centrifuged at 14,000 rpm at 4 °C for 20 min, decanted and 500 µL of 70% cold ethanol added, and centrifuged again. The supernatant was decanted. This last step was repeated once more. The DNA button was dried and resuspended in 50 µL of TE. Finally, 3 µL of RNase (10 mg/mL) was added. The tubes were incubated at 37 °C for 1 h and finally at 60 °C for 10 min. The concentration of extracted DNA was quantified in a microdrop scanning UV–VIS spectrophotometer. The DNA was stored at − 20 °C until use.

Amplification of DNA samples was carried out by PCR. The mixture for a 50 µL reaction was made with universal oligonucleotides for bacteria: 27F/1385R (GAGTTTGAT CCTGGCTA / CGGTGTGTTCAAGGCCC) or the oligos specific for the *Bacillus* genus: rpoB 1206F/rpoB 3202R (ATCGAAACGCCTGAAGGTCCAAACAT / ACACCC TTGTTACCGTGACGACC), in a concentration of 1 µL (10 µM), 1 µL of DNTPs 10 mM, 5 µL of regulator 10X, 2.5 µL of $MgCl_2$ (0.125 mM), 0.2 µL of Taq DNA polymerase, and nuclease-free water for a final volume of 50 µL per reaction. Finally, the mixture was added to each tube, and the DNA (50 ng/µL) was added.

The reaction was carried out in a thermocycler with the following program for oligonucleotides 27F/1385R: 95 °C for 2 min, 29 cycles of 95° for 1 min, 58 °C for 1 min, 72 °C for 1: 30 min, and 72 °C for 10 min. For specific genus *Bacillus* oligonucleotides, the following program was used: 95 °C for 3 min, 35 cycles of: 95 °C for 20 s, 55.9 °C for 30 s, 72 °C for 90 min, and 72 °C for 5 min.

The quality of the PCR product was evaluated by electrophoresis on a 1% agarose gel. A molecular weight marker of 3000 base pairs was used. The PCR products were sent to Macrogen (Korea). The sequences were edited with FinchTV program, subsequently assembled with the Codon Code Aligner program, and compared with other sequences in the GenBank database: https://www.ncbi.nlm.nih.gov/BLAST/.

Results

One hundred and sixty-one strains of bacteria were isolated, of which 23 showed the degrading activity of C6HSL signaling molecules (14%).

Table 1 lists the active strains and their characteristics: number of active strains, number of crude extracts, closer species, the percentage of identity related to molecular identification, Gram, the collection site, the type of sample, and the temperature of incubation to which they grew optimally.

The largest number of strains that degraded C6HSL molecules are Gram-positive bacteria of the genus *Bacillus*; most were identified as *Bacillus licheniformis*.

Of the thermotolerant strains, 23% had the degrading activity of C6HSL molecules. Of 55 isolated strains, 13 were active. Mesophilic strains also showed activity. Of the 106 strains isolated, only 10 of them showed activity, which represents 9.43%.

The strains that grew at 60 °C and showed activity were also incubated at 35 °C to establish whether they were

Table 1 Degrading strains of signaling molecules and main characteristics

ID	Species	% iden	Gram	Collection site	Sample	T°
1	*Vibrio alginolyticus*	99	–	Mapachitos	Water	35 °C
2	*V. alginolyticus*	100	–	Santispac	Sediment	35 °C
3	*V. alginolyticus*	99	–	Mapachitos	Water	35 °C
4	*Bacillus licheniformis*	100	+	Santispac	Sediment	35 °C
5	*B. firmus*	100	+	Mapachitos	Sediment	35 °C
6	*Virgibacillus salarius*	99	+	El Sargento	Sediment	35 °C
7	*B. licheniformis*	99	+	Mapachitos	Sediment	35 °C
8	*V. alginolyticus*	99	–	Santispac	Water	35 °C
9	*B. licheniformis*	100	+	Mapachitos	Water	35 °C
10	*B. oceanisediminis*	100	+	Mapachitos	Sediment	35 °C
11	Unidentified		–	Mapachitos	Water	60 °C
12	*Bacillus aerius*	99	+	Santispac	Sediment	60 °C
13	*B. licheniformis*	100	+	El Sargento	Sediment	60 °C
14	*B. licheniformis*	100	+	Mapachitos	Water	60 °C
15	*B. paralicheniformis*	100	+	El Sargento	Sediment	60 °C
16	*B. licheniformis*	100	+	El Sargento	Sediment	60 °C
17	*Bacillus sonorensis*	99	+	Mapachitos	Sediment	60 °C
18	*B. licheniformis*	100	+	El Sargento	Sediment	60 °C
19	*B. licheniformis*	100	+	El Sargento	Sediment	60 °C
20	*B. licheniformis*	100	+	Mapachitos	Water	60 °C
21	*B. licheniformis*	100	+	El Sargento	Sediment	60 °C
22	*B. paralicheniformis*	99	+	Santispac	Sediment	60 °C
23	*Brevibacillus thermoruber*	98	+	El Sargento	Sediment	60 °C

thermophilic or thermotolerant, and the results showed that they are thermotolerant since all of them also grew at 35 °C.

Biofilm inhibitory activity of extracts

The results of the activity of the 23 extracts on the different biofilm-forming strains are shown in Figs. 1, 2, and 3.

All extracts showed activity against the target strains since they somewhat inhibited the formation of biofilm of one or more strains. Most of them inhibited the biofilm formation of the strains of interest by 50% or more.

All tested extracts inhibited the biofilm formation of *P. aeruginosa* PAO1 by more than 50% in the highest concentration (1000 µg/mL). Extracts with the highest activity were as follows: 13- *B. licheniformis* (98%), 15- *B. paralicheniformis* (94%), 10- *B. oceanisediminis* (92%), 14- *B. licheniformis* (91%), 17- *B. sonorensis* (91%), 9- *B. licheniformis* (90%), and 21- *B. licheniformis* (90%), inhibiting almost 100% the formation of the biofilm (Fig. 1a).

Against *Aeromonas caviae* ScH3, the most active were 10- *B. oceanisediminis* (97%), 7- *B. licheniformis* (92%), 9- *B. licheniformis* (91%), 15- *B. paralicheniformis* (90%), and 3- *V. alginolyticus* (82%) also at the maximum concentration tested (Fig. 1b).

Those that showed better inhibitory activity against *V. parahaemolyticus* were 22- *Bacillus paralicheniformis* (88.7%), 19- *B. licheniformis* (86.4%), and 23- *Brevibacillus thermoruber* (81%), at a concentration of 1000 µg/mL (Fig. 2).

The inhibitory activity of the extracts against marine strains related in biofouling processes, *Virgibacillus* sp. C29 and *Vibrio alginolyticus* C96, was lower than with the

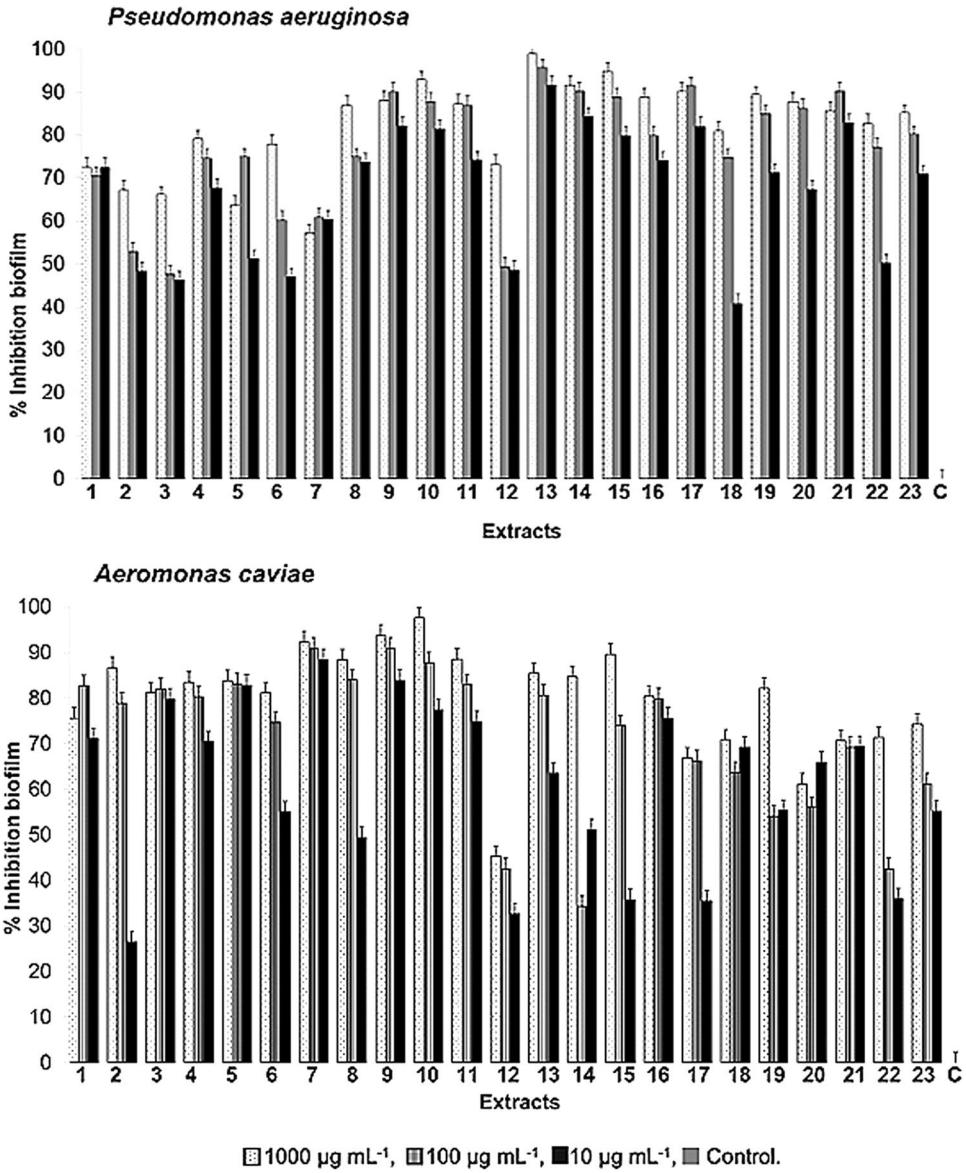

Fig. 1 Biofilm inhibitory activity of bacteria isolated from hydrothermal vents against human pathogenic strains, *Pseudomonas aeruginosa* (**a**) and *Aeromonas caviae* (**b**)

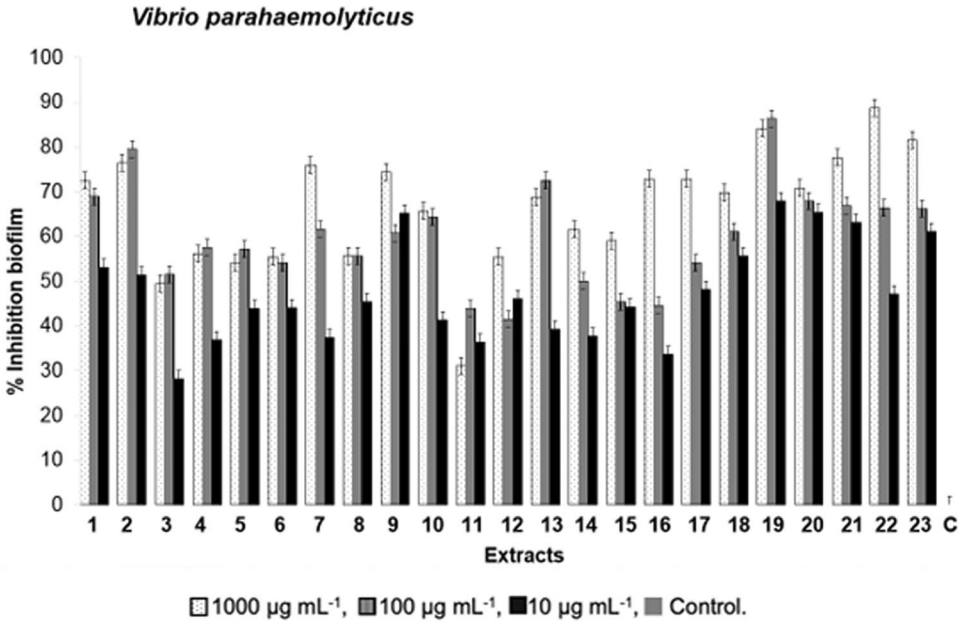

Fig. 2 Biofilm inhibitory activity of bacteria isolated from hydrothermal vents against the pathogenic strain *Vibrio parahaemolyticus*

assay of the pathogenic strains. The concentration with the best inhibitory activity against *V. alginolyticus* C96 was at 1000 µg/mL. The extracts with the highest inhibitory activity were 3- *V. alginolyticus* (75%), 6- *Virgibacillus salarius* (79%), 21- *B. licheniformis* (74%), and 22- *B. paralicheniformis* (80%) (Fig. 3a). While extracts of 10- *Bacillus oceanisediminis* (98.8%) and 4- *B. licheniformis* (98%) at a concentration of 1000 µg/mL inhibited the biofilm formation of *Virgibacillus* sp. C29 (Fig. 3b).

Extract 22- *B. paralicheniformis* inhibited the formation of biofilms of all strains with which the assays were performed with great efficiency.

According to the previous results, extracts that showed the best percentages of inhibition and that were active against biofilm-forming strains tested were selected to carry out the kinetics of growth and biofilm inhibition (Figs. 4, 5). The kinetics of the two marine biofilm-forming strains exposed to the extracts of strains 13- *B. licheniformis*, 21- *B. licheniformis*, 10-*B. oceanisediminis,* and 22- *B. paralicheniformis* are shown in Fig. 4b. With both strains, it is observed that the extracts do not inhibit their growth (Fig. 4a) but do inhibit the formation of the biofilm (Fig. 4b). With *Virgibacillus* sp. after 30 h, an increase in growth is observed and this is also reflected in greater adhered biofilm, which was no longer completely inhibited by extracts; however, it is observed that it is less than the control without extract. An inhibition of biofilm formation is observed from the first hours, since the attachment process begins. On the other hand, with *Vibrio alginolyticus* an inhibition of biofilm formation was observed during all the hours evaluated, with strains 21 and 22 a decrease is observed after 24 h. For both target strains, the time of greatest biofilm formation was at 30 h (Fig. 4b).

Against the two pathogenic strains evaluated, the extracts showed no effects on their growth (Fig. 5a), but an inhibition in the formation of biofilms, both for *Pseudomonas aeruginosa* and *Aeromonas caviae*, the difference with the control without extract being very remarkable (Fig. 5b). Against *Pseudomonas aeruginosa*, the extracts seem to inhibit the formation of the biofilm after 24 h, which is when the increase of biofilm, is observed in the control without extract.

Discussion

QS seems to play a very important role in converting microcolonies to mature biofilm, which is why QS inhibitors can potentially stop the transition to this stage where bacteria are more tolerant. Also, because it has been found that QS is not essential for bacterial growth, QS inhibitors are expected to cause less selective pressure on bacteria than regular bactericidal treatments. Therefore, the search for new strategies to block this communication system could be more effective antimicrobial alternatives without the immediate risk of developing resistance (Scutera et al. 2014).

It has been reported that AHL-based QS systems are interrupted by several mechanisms including quorum detection inhibitors (QSI), which act as AHL antagonists and interfere with the detection of signal molecules (Givskov et al. 2013; Bhardwaj et al. 2013; Reina et al. 2019).

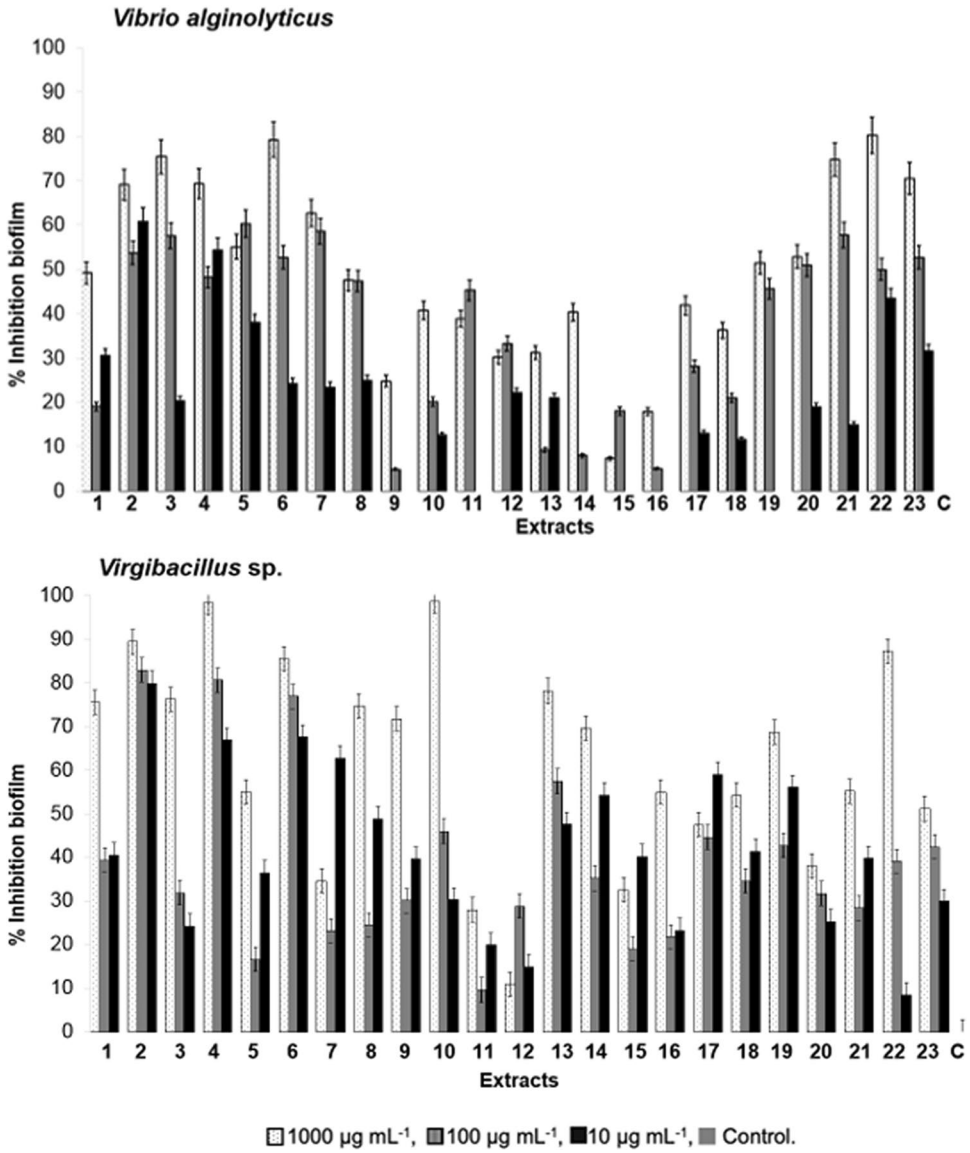

Fig. 3 Biofilm inhibitory activity of bacteria isolated from hydrothermal vents against strains involved in the biofouling process, *Virgibacillus* sp. (**a**) and *Vibrio alginolyticus* (**b**)

Many studies have demonstrated anti-QS activity in a wide range of organisms, such as plant extracts (Bodini et al. 2009; Tolmacheva et al. 2014), fungi (Zhu and Sun 2008; Zhu et al. 2011), and bacteria (Ni et al. 2009; Kalia 2013; Singh et al. 2017). Marine microorganisms are producers of numerous active secondary metabolites and are considered as an important resource in the search for new anti-QS compounds (Dobretsov et al. 2009; Teasdale et al. 2001; Sun et al. 2016; Yaniv et al. 2017; Torres et al. 2019); within marine environments, hydrothermal vents are considered as an underexplored source of new bioactive molecules.

In this study, 161 bacterial strains isolated from three hydrothermal vents were evaluated for their ability to produce QSI compounds and inhibit the formation of biofilms of pathogenic strains and marine bacteria involved in the biofouling process, and of these, 23 showed activity of C6HSL signaling molecules.

Most of the strains that degraded signaling molecules of the AHL type (C6HSL) and showed biofilm inhibitory activity were thermotolerant (23%). Only 9.4% of the mesophilic strains had this activity. In many studies, it is mentioned that enzymes are responsible for the IQS activity (Dong and Zhang 2005; Ng et al. 2011; Kalia and Purohit 2011; Kalia 2013), in the present study it is shown that they are not only enzymes, since the presence of other molecules in the active extracts obtained with ethyl acetate can act as autoinductors. It has been reported that, in similar extracts with the same solvent, IQS properties are found, such as dicetopiperazines, methyl dodecanoic acid, foranosyl borate, farnesoic acid, palmitic acid, and other AHL signaling molecules of different numbers of analogous carbons (Rodelas et al. 1999; Dong and Zhang 2005; Brencic and Winans 2005; Teasdale et al. 2009; Melvin et al. 2016).

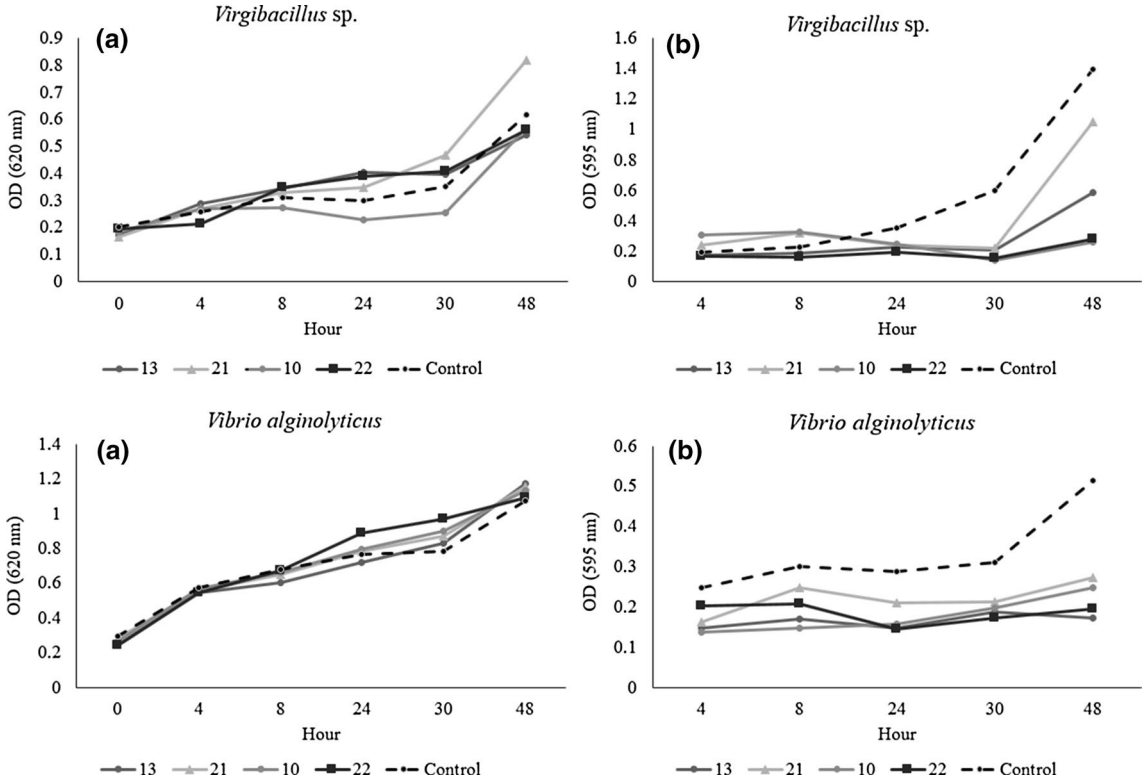

Fig. 4 Effect of *Bacillus* extracts on the **a** growth and **b** inhibition of the biofilm of *Virgibacillus* sp. and *Vibrio alginolyticus* estimated by staining with crystal violet

Similarly, the molecule hordenine, also a tyramine derivative, has been found to be capable of inhibiting QS in *P. aeruginosa* (Zhou et al. 2018). Other molecules associated with tyramine and N-acetyltyramine, such as N-(2′-phenylethyl)-isobutyramide, 3-methyl-N-(2′-phenylethyl)-butyramide (Teasdale et al. 2009), 4-(2-hydroxyethyl)-phenol, and tyrosol acetate (Martínez-Matamoros et al. 2016), have also been found to be capable of inhibiting QS-mediated phenotypes. Some bacteria associated with corals have been reported to produce compounds, such as rhodamine isothiocyanate, that inhibit QS in *C. violaceum* (Song et al. 2018). In this study, 23 strains were selected as QSI-producing bacteria based on their capacity to inhibit the QS system of *C. violaceum* CV026. Interestingly, most of them belong to the genus *Bacillus*.

The genus *Bacillus* stands out among bacteria that presented both autoinducer-degrading activity and inhibition of bacterial biofilm formation. Thermotolerant species, such as mesophylls (*B. licheniformis, B. firmus, B. oceanisedimenis, B. aerius, B. sonorensis,* and *B. paralicheniformis*), are adapted to warm environments and generally have simple nutrient requirements, making them capable of colonizing oligotrophic niches, such as marshes, hot springs, and desert soils (Khiyami et al. 2012). Zeikus (1979) reported that all *Bacillus* species, whether mesophilic or not, contain some degree of thermostability, which is why many of the identified strains of this genus are used in this study, grew at 60 °C. Multiple investigations related to the IQS process and the ability of other bacteria to inhibit biofilms have been published, and this activity is related to the presence of exopolysaccharides (Sayem et al. 2011), some enzymes, such as α-amylase (Kalpana et al. 2012), and biosurfactants (Rivardo et al. 2009), among other compounds. In general, many IQS lactonase enzymes have already been reported in species of the genus *Bacillus*. However, Nithya et al. (2010) demonstrated for the first time that a strain of *B. pumilus* produced an enzyme that is not lactonase and can degrade AHL molecules. The strain also significantly inhibited LasA proteases (76%), LasB elastases (84%), caseinases (70%), pyocyanins (84%), pyoverdins, and biofilm formation (87%) of the same species that were tested in this work, *Pseudomonas aeruginosa* PAO1.

There are studies related to the production of exopolysaccharides in *Brevibacillus thermoruber* (Radchenkova et al. 2011). This species is phylogenetically related to *Bacillus licheniformis* (Yohandini et al. 2015). The strain *Virgibacillus salarius* also showed activity and is also phylogenetically related to the genus *Bacillus* (Hua et al. 2008). It can be inferred that these strains, like the genus *Bacillus*, produce exopolysaccharides, enzymes that degrade signaling

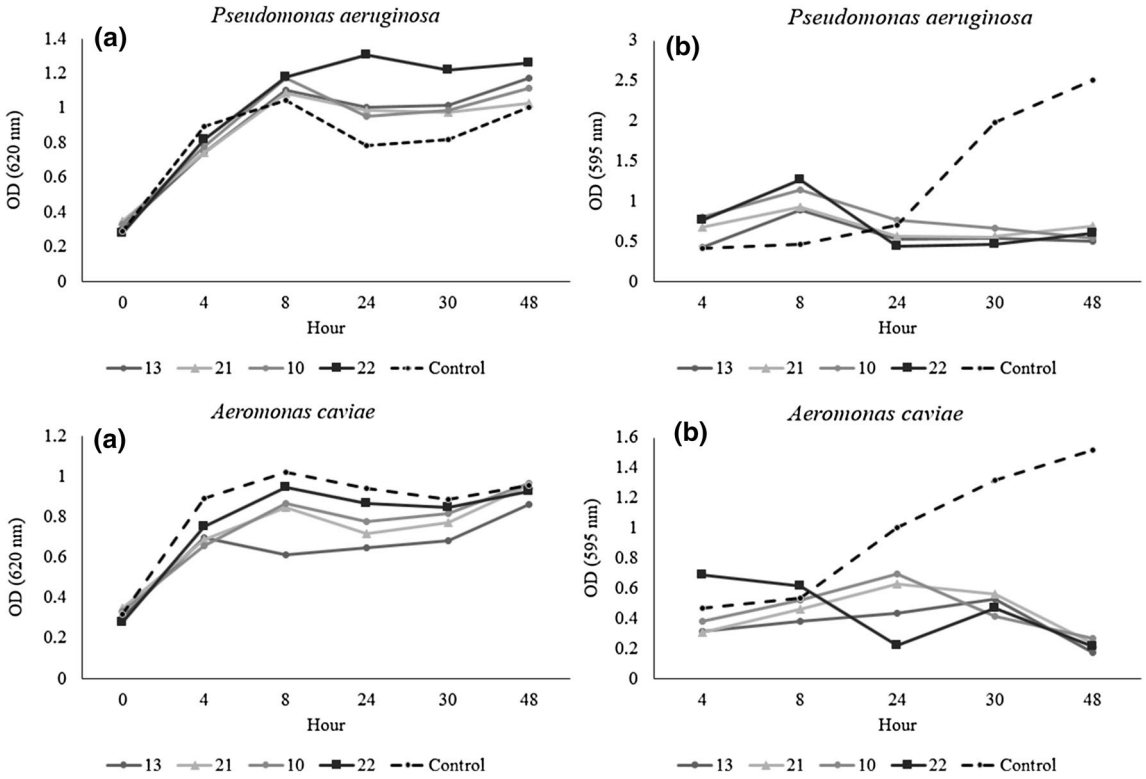

Fig. 5 Effect of *Bacillus* extracts on the **a** growth and **b** inhibition of the biofilm of *Pseudomonas aeruginosa* and *Aeromonas caviae* estimated by staining with crystal violet

molecules and other compounds, such as those mentioned above that are involved in IQS.

Vibrio alginolyticus is another species that demonstrated degrading activity of C6HSL signaling molecules and inhibited the formation of biofilms of certain strains. Its activity may be mainly due to signaling molecules of the 4C-HSL type, such as those detected by Lule (2007) in a species of *Vibrio*. He also obtained liquid–liquid extracts with ethyl acetate as a solvent, and, using a gas chromatograph with a spectrometer of masses (GC/MS), detected palmitic and stearic acids, as well as diketopiperazines. These compounds have been identified as bacterial communication inhibitor molecules. It is possible to infer that the extracts we obtained from that genus for this study also contained these compounds.

Leadbetter and Greenberg (2000) mentioned that many members of the Proteobacteria class have QS systems that are based on acyl-homoserine lactone (acyl-HSL) signals, as in this case of *Vibrio alginolyticus* that belongs to the Gammaproteobacteria class. Jiang et al. (2011) published that exopolysaccharides of the strain *Vibrio* sp. QY101 successfully inhibited the biofilm of *Pseudomonas aeruginosa* and has great potential in the design of new therapeutic strategies for bacterial infections associated with biofilms and specifically, to limit the formation of biofilms in permanent medical devices that cause nosocomial diseases.

All crude extracts that were tested in the assays had biofilm inhibitory activity of different strains, either to a lesser or greater extent. However, it was observed that the crude extract with the highest activity against all strains was number 22, performed from a culture of *Bacillus paralicheniformis.* This confirms that the genus *Bacillus* has an important function in inhibiting the formation of biofilms of other strains. The previously mentioned studies on this genus showed that it is enzymes that give it the ability to inhibit the formation of biofilms. This study shows that it is not only the enzymes that have such activities because the method used to make extracts with ethyl acetate did not extract acylases or lactonases. However, there are studies that support the reduction of biofilm of species, such as *P. aeruginosa* PAO1 by anti-QS extracts, could be associated with analogs of AHL-type signaling molecules, which could compete for their receptor, leading to a receptor biofilm inhibition (Ma et al. 2018).

The results derived from the biofilm formation inhibition assays showed that the crude extracts showed higher activity at higher concentrations and also greater activity against pathogenic strains. A possible explanation for this finding is that marine bacteria could generate resistance to

compounds produced by other bacteria in their same natural habitat, while terrestrial pathogens do not because they are not in frequent contact with compounds produced by the marine bacteria isolated in this study. Recent genetic and genomic studies have shown that a biofilm is formed in multiple steps (Watnick et al. 1999). In some organisms. it requires intercellular signaling (Shih and Huang, 2002) and a specific genetic profile of gene transcription is needed for biofilm formation (Whiteley et al. 2001; Schembri et al. 2003; Beloin et al. 2004).

On the other hand, studies with other microorganisms have revealed that fimbriae or pili are important for biofilm formation by *Salmonella enteritidis* (Austin et al. 1998), *Pseudomonas aeruginosa* (O´Toole and Kolter 1998), *Staphylococcus epidermidis* (Heilmann et al. 1997), and *Escherichia coli* (Pratt and Kolter 1998). These protein structures recognize a wide range of molecular targets, allowing the bacteria to interact with various surfaces (Klemm and Schembri 2000). The biofilm formed by our strain under aerobic growth conditions was in most of the strains, more intense in the air–liquid interface; however, in *Aeromonas caviae, Vibrio alginolyticus,* and *Virgibacillus* sp. which are facultative anaerobes, biofilm was also observed throughout of the wells, which is an indicator of the metabolism of the strains used. In other works with cell agglutination experiments under anaerobic growth conditions, they have suggested that additional elements in addition to type I fimbriae could be involved in biofilm formation in the interface (Cabellos et al. 2006).

According to the result of the growth kinetics, it was observed that the extracts do not inhibit the growth of the target strains, in comparison with the results of the kinetics of biofilm formation, where an inhibition was found in all cases the first hours, so it is suggested that the extracts have the ability to prevent the adhesion of the cells during the adhesion process, which increases at 24 h for the case of pathogenic strains and at 30 h for the strains marine (*Virgibacillus* sp. and *Vibrio alginolyticus*), showing maximum biofilm formation at 48 h. In microorganisms, one of the main virulence factors is the formation of biofilms, which facilitates adaptation to adverse environmental conditions, and this could also be an explanation, since at 48 h a competition for space and / or nutrients would begin.

Conclusion

Marine bacteria isolated from shallow hydrothermal systems, both mesophilic and thermotolerant, produce compounds that degrade signaling molecules of the AHL type.

Mostly species of the genus *Bacillus*, inhibiting to a greater extent the formation of biofilms of pathogenic bacteria, contrary to those of marine bacteria related to biofouling processes, possibly accustomed to the compounds produced by the active marine bacteria that were tested.

The scientific evidence of this work reinforces the idea that shallow hydrothermal systems are an accessible source of thermotolerant and mesophilic microorganisms with compounds that have biotechnological potential to inhibit bacterial biofilms.

Acknowledgements This work was financed in part by SIP-IPN grant. Authors are BEIFI/CONACyT/COFAA/EDI fellows.

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