ORIGINAL ARTICLE

Antimicrobial properties of moderately halotolerant bacteria from cenotes of the Yucatan peninsula

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Keywords
antimicrobial, bacteria, bacteriocin, biosurfactant, cenote, halotolerant, siderophore.

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2006/1690: received 4 December 2006, revised 19 April 2007 and accepted 21 April 2007

doi:10.1111/j.1472-765X.2007.02185.x

Abstract

Aims: Isolation and antimicrobial evaluation of aquatic bacterial strains from two cenotes.

Methods and Results: A total of 258 bacterial strains were isolated from the water and sediment of two cenotes in the Yucatan peninsula, all of which were screened against six pathogenic micro-organisms. Antimicrobial activity was detected in 46 of the isolated strains against at least one of the target strains tested. Antimicrobially active isolates were identified as: *Aeromonas*, *Bacillus*, *Burkholderia*, *Photobacterium*, *Pseudomonas*, *Serratia*, *Shewanella*, *Stenotrophomonas* genera, and 13 remained unidentified. All antimicrobially active strains were able to grow in salt medium at a concentration of 75 g l^{-1}, thus classifying as moderately halotolerant bacteria. Most of the antimicrobially active strains exhibited a broad action spectrum, where 61% was because of uncharacterized antimicrobial substances, 25% because of bacteriocins and 13% because of siderophores. Ten strains were able to biosynthesize biosurfactant metabolites.

Conclusions: Native bacteria from the Yucatan peninsula showed an interesting antimicrobial activity, diverse mode of action and moderate halotolerance to salt.

Significance and Impact of the Study: This is the first report on bacterial isolates from cenotes of the Yucatan peninsula and their antimicrobial characterization, with great potential for future biotechnological applications.

Introduction

Microbial secondary metabolites have been recognized as a major source of compounds endowed with ingenious structures and potent biological activities (Donadio *et al.* 2002). During the past 60 years, several of these have revolutionized the medical field by providing the cure for formerly life-threatening diseases. However, the incessant search for new metabolites continues because of the fact that some pathogenic micro-organisms have recently emerged and others are virtually unresponsive to antibiotics. In fact, no novel or effective chemical antibiotics have been discovered during the last few decades (Hancock and Chapelle 1999). The urgent need for new classes of antibiotics is evident.

One of the strategies followed in this search is the isolation of micro-organisms from nonexplored regions, such as bacteria from freshwaters (Borris 1996). From that point of view, recent estimates suggest that as little as 0·1% of existing bacteria have been identified, and only a small fraction has been examined for metabolite profile (Lange 1996). Some of these scarcely studied regions in the tropical zones are the cenotes (sinkholes) of the Yucatan peninsula. Cenotes are peculiar aquatic ecosystems connected to underground waters and located in a calcareous platform characterized by the absence of rivers or lagoons (Lopéz-Adrian and Herrera-Silviera 1994). The microbial diversity reported in these locations is limited to sulfate-reducing bacteria and S-oxidizing bacteria (Martinez *et al.* 1998; Stoessell *et al.* 2002).

The aim of this work is to carry out further studies on the isolation of native bacteria from aquatic environments of the Yucatan peninsula, and explore their antagonistic

properties against micro-organisms pathogenic to humans and plants.

Materials and methods

Isolation

Samples were collected from the water and sediment of the cenotes Temozon ('La Noria' 1GQ 022 90 74 UTM 23 32 28), and X'Kan ho ho che (Dzibilchaltun Eco-archeological park) close to Merida City, Yucatan, Mexico. These micro-organisms were isolated after a 1 : 1000 dilution with sterile cenote water and grown on 10% tryptic soy agar (TSA) medium (pH 8·5), followed by incubation for 24–48 h at 25°C. All strains were kept frozen at −80°C in glycerol (2·5% v/v).

Bacterial targets

Six target organisms were used: *Candida albicans* (ATCC-10231), *Bacillus subtilis* (ATCC-6633), *Staphylococcus aureus* (ATCC-6536), *Pseudomonas syringae* pathovar. *pisi* (ATCC-11043), *Xanthomonas campestris* pathovar. *carotae* (ATCC-10547) and *Erwinia carotovora* subp. *carotovorum* (ATCC-138).

Antimicrobial activity assay

All the bacteria isolated (258 strains) were tested on TSA plates using an adaptation of Messi's technique (Messi *et al.* 2003). The bacterial strains were spotted with an applicator onto TSA plates, previously inoculated with each target strain ($1{\cdot}5 \times 10^6$ CFU ml^{-1}). After 20 h of incubation at 25°C, antagonism was quantified by a clear inhibition zone around the spot of the bacterial strains. Amykacine was used as positive control and each target strain as negative control. Each test was carried out in triplicate. All antimicrobially active isolates were identified and screened for siderophores, biosurfactant and bacteriocin detection.

Identification of antimicrobially active isolates

Antimicrobially active isolates (46) were identified by observing the appearance and consistency of the colonies, Gram's stain, oxidase production and by mode of attack on glucose (fermenting or nonfermenting bacteria) selecting the test system API 20NE or API 20E, and other complementary tests.

Siderophores detection

Antimicrobially active isolates were spot-inoculated onto CAS agar plates [Chrome azurol S, iron (III), hexadecyltrimethylamonium bromide], and incubated at 25°C for 48 h. Siderophore-production strains were detected by an orange-yellow halo around the spot (Schwyn and Neilands 1987).

The ability to produce antimicrobial siderophores was screened by the agar diffusion assay using TSA medium, previously inoculated with each target bacteria at $1{\cdot}5 \times 10^6$ CFU ml^{-1}, with or without iron (ferric chloride 0·3%). Antagonistic activity was detected by partial or total elimination of the inhibitory halo with iron in the medium. Additionally, antimicrobially active strains were grown in different iron-chelating test compounds in tryptic soy broth (TSB) medium (Vachée *et al.* 1997).

Biosurfactant detection

Drop collapse method

The drop-collapse technique was carried out following indications described in the literature for qualitative (Mineral oil) and quantitative (Pennzoil®) assays. Fifteen replicates of each strain were tested (Bodour and Miller-Maier 1998).

Oil spreading technique

This technique was carried out as described in the literature (Youssef *et al.* 2004). Ten replicates of each antimicrobially active strain were measured.

Bacteriocin detection

Bacteriocin production was assessed by the effect of protease on extracellular metabolites produced by antimicrobially active strains in agar diffusion assay. Nitrocellulose filter disks (0·45 μm) were transferred into buffer TRIS pH 7·5, with or without pronase E (10 g l^{-1}) and incubated for 3 h at 30°C. The disks were then placed on TSA medium, previously inoculated with each target bacteria at $1{\cdot}5 \times 10^6$ CFU ml^{-1}. Antimicrobially active strains were spot-inoculated onto the disks, and maintained at 25°C for 24 h. If inhibitory substances were inactivated by pronase E, then the inhibitory zone decreased or disappeared in the indicator lawn around the filter (Benkerroum *et al.* 1993).

Tolerance to sodium chloride

A sample of the strains (46 antimicrobially active and 39 nonactive) were cultivated in TSA medium enriched with different sodium chloride concentrations (15, 25, 35, 50, 75, 100, 150 and 200 g l^{-1}), and maintained at 25°C. After 24 h, the strains that were able to grow were observed and classified as moderately halotolerant (≥75 g l^{-1} NaCl), and nontolerant (nongrowth) (Hacěne *et al.* 2004).

Results

Isolation and identification of antimicrobially active strains

The samples collected during explorations in two cenotes resulted in 258 (240 from water and 18 from sediment samples) pure isolates; 222 were detected as gram-negative bacteria. Screening for antimicrobial activity of all isolates indicated that only 46 were active and these were identified and screened for siderophores, biosurfactant and bacteriocin detection. Twenty-nine isolates were identified at species level as *Aeromonas salmonicida*, *Bacillus cereus*, *Burkholderia cepacia*, *Burkholderia gladioli*, *Pseudomonas luteola*, *Pseudomonas aeruginosa*, *Serratia plymuthica*, *Shewanella putrefaciens* and *Stenotrophomonas maltophilia*. Among these, *Ps. luteola* ($n = 16$) was the most common species isolated, followed by *Burk. cepacia* ($n = 5$). Three strains were identified to the genus level (*Burkholderia*, *Photobacterium* and *Stenotrophomonas*), and 13 active isolates remained unidentified. These corresponded to seven nonfermenting gram-negative and six gram-positive bacteria (Table 1).

Antimicrobial activity assay

In this study, 46 isolates (40 from water and 6 from sediment samples) with inhibitory activity against at least one indicator strain were detected. In general, 34 isolates showed a broad activity spectrum, being able to inhibit gram-negative, gram-positive and/or *C. albicans*, while the remaining 11 strains (*B. cereus*, two isolates of *Burk. cepacia*, *Ps. luteola*, *Photobacterium* sp., *Ser. plymuthica*, *Sten. maltophilia* and four unidentified gram-negative isolates) showed specific activity against only one target.

The greatest antagonistic action was detected against *Ps. syringae*, and *X. campestris* (Table 1). In particular, two isolates, one of *Ps. luteola* and one of *Stenotrophomonas* sp. limited the growth of all target micro-organisms with average inhibition zones between 11 and 30 mm. The highest inhibition zone was 38 mm, displayed by *Ps. luteola* against *Ps. syringae*. It was also observed that *Burk. cepacia*, *Stenotrophomonas* sp., *Shew. putrefaciens* and an unknown isolate (2X83) produced the highest inhibition zones against *Staph. aureus* (21 mm), *X. campestris* (21 mm), *E. carotovora* (18 mm) and *B. subtillis* (20 mm), respectively. Other interesting strains corresponded to an unknown isolate (1T103) which exhibited activity only against *C. albicans* (16 mm).

Siderophores detection and production

The presence of siderophores was detected with CAS medium in 42 of 46 isolates, including all active *Pseudomonas* isolates. This was confirmed by using four different iron-chelating-enriched media. Furthermore, the strains were evaluated in medium with $FeCl_3$, where it was possible to detect 14 cases with partial activity against *Staph. aureus*, *B. subtilis* and *Ps. syringae*. Interestingly, in five cases corresponding to two strains of *Burk. cepacia*, an unidentified gram-positive strain (1X44) against *X. campestris*, bacterial strain 2X83 against *B. subtilis* and *A. salmonicida* against *Ps. syringae* (Table 1), activity was totally eliminated, indicating that the inhibitory activity was only because of siderophores.

Bacteriocin production

The presence of the enzyme pronase E during the antagonistic assay allowed the detection of 26 strains producing proteinaceous inhibitory metabolites within the antimicrobially active isolates. Bacteriocins detected in the present study were often active against, *Ps. syringae* (11), *E. carotovora* (7), *Staph. aureus* (7), *X. campestris* (5) and *B. subtilis* (1) but were inactive against *C. albicans*. Five of them were partially inactivated by proteolytic treatment: one isolate of *Ps. luteola*, and two unidentified strains (1X44, 2X53) against *Ps. syringae*; the same effect was observed in *Stenotrophomonas* sp. and one unidentified isolate (2X52), both against *X. campestris*.

On the other hand, in 23 strains the activity was completely lost because of the presence of the enzyme: eleven isolates of *Ps. luteola*, *Ps. aeruginosa*, two isolates of *Burk. cepacia*, *Burk. gladioli*, *Sten. maltophilia*, *Stenotrophomonas* sp., *Photobacterium* sp. and four unidentified isolates. In general, antagonistic activity of the bacteriocin type was observed against only one target, five strains against two pathogens and two isolates of *Ps. luteola* against three target micro-organisms.

Biosurfactant production

Screening biosurfactant-producing micro-organisms permitted the selection of 10 isolates with good activity using the drop-collapse and oil spreading techniques. These corresponded to two isolates of *Burk. cepacia* and *Burk. gladioli*, two isolates of *Ps. luteola*, *Shew. putrefaciens* and four unidentified isolates. The data detected two excellent biosurfactant producers (T243 and 2X52) with drop diameters between 5·0 and 23 mm.

Tolerance to sodium chloride

The 46 antimicrobially active isolates were able to grow in a medium enriched with NaCl (75 g l^{-1}), many of

Table 1 Inhibitory activity and partial antimicrobial characterization of active isolates from cenotes of the Yucatan peninsula against six target strains

Species		*Staphylococcus aureus*	*Bacillus subtilis*	*Pseudomonas syringae*	*Candida albicans*	*Xanthomonas campestris*	*Erwinia carotovora*
		Mean diameter of inhibition halo, mm (nature of antimicrobial activity)					
Aer. salmonicida	2X71	–	16 (AMS)	15 (S)	–	–	–
Bacillus cereus var. *cereus*	1T41	–	19 (PS)	–	–	–	–
Burkholderia cepacia	1T31	10 (BLS)	18 (PS)	–	14 (AMS)	16 (BLS)	–
	2T51	21 (PS)	17 (PS)	–	–	–	–
	2X89	–	–	–	–	14 (S)	–
	2X109	–	–	–	–	17 (S)	–
	1X03	19 (PS)	16 (AMS)	16 (B)	–	–	–
Burkholderia gladioli	1X02	11 (BLS)	16 (AMS)	16 (BLS)	–	–	–
Burkholderia sp.	1X41	13 (AMS)	–	26 (AMS)	–	13 (AMS)	–
Photobacterium sp.	2X104	–	–	18 (B)	–	–	–
Pseudomonas aeruginosa	2T16	18 (AMS)	14 (AMS)	15 (B)	–	–	–
	2T17	18 (PS)	18 (AMS)	–	–	–	–
Pseudomonas luteola	1T121	–	14 (AMS)	16 (B)	–	14 (AMS)	13 (AMS)
	1T122	–	14 (AMS)	16 (AMS)	–	7 (AMS)	11 (B)
	1T131	14 (AMS)	–	32 (PS)	–	14 (AMS)	10 (AMS)
	1T102	11 (AMS)	–	–	–	7 (AMS)	–
	1T17	18 (BLS)	15 (AMS)	19 (BLS)	–	18 (BLS)	11 (AMS)
	1X42	14 (AMS)	–	38 (PS)	–	16 (AMS)	–
	1X43	13 (AMS)	–	23 (AMS)	–	11 (AMS)	12 (AMS)
	1X45	–	15 (AMS)	–	–	–	10 (B)
	1T23	–	16 (AMS)	17 (PB)	–	–	10 (B)
	1T22	–	–	20 (B)	–	12 (B)	11 (B)
	2T06	–	–	15 (B)	–	–	–
	1T05	16 (AMS)	15 (AMS)	29 (AMS)	11 (AMS)	16 (AMS)	11 (AMS)
	1T41	–	–	25 (AMS)	11 (AMS)	17 (AMS)	13 (B)
	1T242	13 (BLS)	–	32 (PS)	11 (AMS)	16 (AMS)	11 (AMS)
	2X41	14 (AMS)	–	25 (PS)	14 (AMS)	17 (AMS)	14 (B)
	2X24	8 (BLS)	16 (AMS)	15 (PS)	–	17 (AMS)	15 (AMS)
Serratia plymuthica	1T23	–	15·5 (AMS)	–	–	–	–
Shewanella putrefaciens	2T41	–	13 (AMS)	18 (AMS)	–	12 (AMS)	18 (AMS)
Stenotrophomonas maltophilia	1X25	–	14 (BLS)	–	–	–	–
Stenotrophomonas sp.	1T202	14 (AMS)	15 (AMS)	30 (PS)	11 (AMS)	21 (PB)	13 (AMS)
	1T16	–	18 (AMS)	28 (B)	–	16 (B)	16 (AMS)
Gram-negative	1T101	15 (AMS)	–	24 (B)	7 (AMS)	18 (AMS)	–
	1T103	–	–	–	16 (AMS)	–	–
	1T18	14 (BLS)	15 (AMS)	18 (BLS)	–	16 (AMS)	11 (AMS)
	1T21	–	17 (AMS)	–	–	15 (B)	–
	1T04	14 (AMS)	–	28 (AMS)	10 (AMS)	16 (AMS)	12 (AMS)
	1T243	13 (BLS)	–	24 (AMS)	11 (AMS)	18 (AMS)	12 (AMS)
	1T32	11 (PS)	16 (AMS)	–	–	11 (AMS)	–
Gram-positive	1X44	–	–	18 (PBLS)	–	9 (S)	7 (BLS)
	2X83	–	20 (S)	–	–	–	–
	2X52	–	15 (AMS)	26 (PS)	7 (AMS)	18 (PBLS)	–
	2X53	13 (AMS)	14·5 (AMS)	30 (PBLS)	–	10 (AMS)	–
	2X103	–	12 (AMS)	–	–	–	–
	2X69	–	15 (AMS)	–	–	–	–

–, Nonactive; (AMS), antimicrobial substances; (B), loss of activity by bacteriocin; (S), loss of activity by siderophores; (BLS), bacteriocin-like substance; (PB), partial loss of activity by bacteriocin; (PBLS), partial loss of activity by bacteriocin-like substance; (PS), partial loss of activity by siderophores.

them at concentrations up to 150 g l^{-1} of salt. These were *Pseudomonas* ($n = 10$), *Burkholderia* ($n = 2$) *Stenotrophomonas* sp. ($n = 1$), gram-negative unidentified isolates ($n = 6$) and one gram-positive isolate. In particular, two *Ps. luteola*, two *Burk. cepacia* and *Photobacterium* sp. isolates showed tolerance up to 200 g l^{-1}.

Discussion

Preliminary explorations of the aquatic bacteria from two cenotes led to the isolation of 258 strains. Gram's stain test indicated that the majority of the isolates were gram-negative bacteria (86%) as reported for other underground waters (Chapelle 2001; Messi *et al.* 2003). These antimicrobially active strains were submitted to identification tests, classifying them as *Aeromonas*, *Bacillus*, *Burkholderia*, *Photobacterium*, *Pseudomonas*, *Serratia*, *Shewanella* and *Stenotrophomonas*. The majority of these genera are reported as the most common heterotrophic bacteria from aquatic environments, such as ground and mineral fresh waters (Vacheé *et al.* 1997; Leclerc 1998). An exception is observed with *Photobacterium* genus, which has been isolated mainly from marine origins (Holt *et al.* 2000), both cenotes investigated are close to the sea suggesting that they could possess a halocline. This effect is possible when a layer of saltwater is present below the freshwater and a meromixis can take place. It is not surprising therefore, that some organisms have evolved from marine ancestors (Schmitter-Soto *et al.* 2002). These antecedents led to the assessment of halotolerance of the isolates to salt. As expected, isolates from cenotes showed an ability to grow in the absence or presence of salt, being classified as moderately halotolerant (Ventosa *et al.* 1998).

Among the 34 isolates detected with a broad activity spectrum, nine were able to inhibit the growth of gram-negative, gram-positive bacteria and *C. albicans*. Excel isolates detected in this study were three *Ps. luteola* strains; however, there have been no previous reports of antimicrobial activity. In general, *Pseudomonas* genus is recognized as a greater producer of active secondary metabolites (Vachée *et al.* 1997).

The presence of antagonistic siderophores and bacteriocins was evaluated in the 46 antimicrobially active isolates, and a search for biosurfactant compounds was also carried out in order to determine the nature of the antimicrobial substances (AMS). Results showed that although a large bacterial population produced siderophores, antagonistic activity mediated by siderophores was detected in only five tests: two *Burk. cepacia* and *A. salmonicida* isolates, together with two unidentified strains. In the literature, the nonpeptides siderophores cepabactin, cepacin, and ornibactin have been described from *Burkholderia* spp. (Kang *et al.* 1998; Berg *et al.* 2005); and aerobactin from *Aeromonas* sp. (Chart and Trust 1983). Micro-organisms with efficient siderophores and greater affinity to iron have an ecological advantage over those without these features. This is characteristic of aquatic oligotrophic environments (Martinez *et al.* 2003).

The production of proteinaceous substances was corroborated in 50% of the active isolates. This showed partial or total loss of the inhibition halo around the indicator strain, in the presence of pronase E. This behaviour was observed in 14 isolates (Table 1) against closely related species, indicating the presence of bacteriocins (Jack *et al.* 1995). A marked activity by bacteriocins was more frequently observed against *Ps. syringae* (50%), and *E. carotovora* (25%). In particular, activity by bacteriocins as a unique mode of action was detected in two strains of *Ps. luteola*, and *Photobacterium* sp. Nine isolates with proteinaceous metabolites exhibited a broad inhibition spectrum, with activity against other species taxonomically remote, suggesting that these antagonistic metabolites are bacteriocin-like substances (BLS). Specifically one isolate of *Sten. malthophilia*, and an unknown gram-positive isolate showed this mechanism of action, which could regulate population dynamics in bacterial ecosystems helping the micro-organism in the competition for the colonization of their environment (Messi *et al.* 2003; Motta *et al.* 2004).

In parallel, active isolates were evaluated by the biosurfactant assay. Four excel candidates producing biosurfactant molecules were detected. The composition, structure and properties of a variety of surfactants produced by bacteria, yeasts and fungi have been described as having antibiotic properties by disrupting micro-organism membranes in competition for food (Banat 1995; Al-Tahhan *et al.* 2000).

In general, antimicrobial activity displayed by isolates from cenotes against six target micro-organisms was produced mainly by bacteriocins or BLS, followed by siderophores and the rest of the observed activity was produced by other kinds not yet characterized as AMS (Table 1). These results differ from those reported in other aquatic bacteria environments where *Pseudomonas* genus was the dominant biota and their mode of action was by siderophores (Vachée *et al.* 1997).

This work constitutes the first contribution in the isolation of autochthonous bacterial species from cenotes of the Yucatan peninsula. Results indicate that microbiota isolated from unique environments can be an interesting alternative in the research of novel compounds for biotechnological applications.

The identification of the most promissory strains by molecular techniques and secondary metabolites isolation are currently in progress.

Acknowledgements

The authors thank Maribel Gamboa-Angulo, Manuela Reyes-Estebanez, Leticia Medina-Baizabal and Una Mulhall for their valuable technical assistance. The authors are also grateful to Miguel Rosado-Vallado for his helpful discussions, Andrés Quijano for providing some target

strains. This research was supported by CONACyT (Project No. 45794) and CONACyT Graduate Student Fellowship S.C.R.G. (84426).

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