Original Research Article

Bioprospecting for culturable actinobacteria with antimicrobial properties isolated from rivers in Colombian Orinoquia

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Abstract

Purpose: To Isolate and characterize Actinobacteria with antimicrobial activity from Guaviare River (Colombia).
Methods: Water and sediment samples were collected from Guaviare River. Direct plating, heat and $CaCO_3$ methods were used to isolate Actinobacteria. Six bacterial strains were tested using T-Streak method: Escherichia coli ATCC 23724, Staphylococus aureus ATCC 25923, Acinetobacter baumannii ATCC 19606, Bacillus subtilis ATCC 21556, Klebsiella pneumoniae ATCC 700603, Chromobacterium violaceum ATCC 31532. Strains of Fusarium sp. H24, Trichoderma harzianum H5 and Colletotrichum gloeosporioides were tested using Kirby-Bauer method. Isolates with high antimicrobial activity were selected for further taxonomic identification.
Results: A total of 374 actinobacteria isolates were obtained. Seven isolates exhibited high antimicrobial activity (p < 0.05) and were confirmed as members of Streptomycetaceae family. Of these, three isolates showed differential phenotypic and genotypic profiles, indicating that they may represent new species.
Conclusions: To date, this is the first study of this type in Colombian Orinoquia and indicates that this promising source of Actinobacteria from aquatic sediments with the ability to produce antimicrobial secondary metabolites.

Keywords: Actinobacteria, Antimicrobial activity, Secondary metabolites, Streptomycetaceae, Phenotypic and genotypic profiles, Colombian Orinoquia

INTRODUCTION

Actinobacteria are a predominant source of antibiotics [1,2] with *Streptomyces* as the major source of bioactive compounds [3]. *Actinobacteria* are found in soils although their isolation has focused on other unexplored habitats [2]. Aquatic habitats like rivers have been poorly explored as a source of *Actinobacteria* with biological activity, despite the fact that river environments can be considered a reservoir of potentially active compounds [4]. The aim of this study was to isolate and to identify *Actinobacteria* from water and sediments samples of Guaviare river (Colombia) and

evaluated their ability to produce natural products with antimicrobial activity.

EXPERIMENTAL

Sample collection

Water and sediment samples were collected in May 2014 from five different locations in Guaviare River (Colombia): location 1 (2°36'19.2"N 72°42'26.2"W), location 2 (2°35'24.3"N 72°41'17.2"W), location 3 (2°34'51.6"N 72°41'34.7"W), location 4 (2°33'59.4"N 72°41'43.8"W), location 5 (2°34'51.4"N 72°39'53.6"W).

Isolation of Actinobacteria

A direct plating method, a dilute heat method, and a $CaCO_3$ enrichment method were used for isolation. For direct plating, 1 g or 1 mL of sediment/water were spread directly. For the dilute heat method 1 g or 1 mL of sediment/water was diluted in 10 mL sterile filtered river water and heated at 55 ☐C for 6 min. For $CaCO_3$ method 1 g of $CaCO_3$ was mixed with 5 g sample and incubated at 45 ☐C for 1 h [5]. Each sample from the three pretreatments was then serially diluted to 10^{-3} and aliquots (100 µL) were spread on oatmeal agar (ISP3), starch casein agar (SCA) and starch casein nitrate agar (SC), supplemented with cycloheximide (50 µl/mL) and nalidixic acid (10 µl/mL). Plates were incubated at 30 ☐C for 8 days. Colonies were selected based on morphological features and were archived in 40 % (v/v) glycerol at -70 ☐C.

Screening for antimicrobial activity

Production of antimicrobial substances and quorum sensing inhibition assay by all isolates was assessed using the T-streak method. Antibiosis was indicated by visually observable growth inhibition of the target organisms, and results were recorded as follows: ++ (complete inhibition), + (partial inhibition), – (no inhibition).

Antifungal activity

Antifungal activities were determined using the Kirby-Bauer method. Inhibition zones were recorded as follows: diameter > 20 mm: +++ (good activity), diameter between 10-20: ++ (moderate activity), diameter < 10 mm: + (weak activity) and – (no activity).

Macroscopic and microscopic features

For isolates with high antimicrobial activity, different culture media were used. Media:

tryptone/yeast extract agar (ISP1), malt/yeast extract agar (ISP2), oatmeal agar (ISP3), inorganic salts starch agar (ISP4), glycerol asparagine agar (ISP5) and starch casein agar SCA (Table 2).

Carbon source profiles

Isolates were grown in 12 well microplates with 5 mL basal agar [$(NH_4)_2SO_4$ 2.64 g/L, KH_2PO_4 2.38 g/L, K_2HPO_4 4.31 g/L, $MgSO_4$ x $7H_2O$ 1.0 g/L and 1.0 ml/L trace element solution ($CuSO_4$ x $5H_2O$ 0.64 g/L, $FeSO_4$ x $7H_2O$ 0.11 g/L, $ZnSO_4$ x $7H_2O$ 0.15 g/L, $MnCl_2$ x $4H_2O$)], supplemented with 1 % (w/v) of a selected carbon source. Lactose, galactose, maltose, ribose, sucrose, xylose, inositol, fructose and carboxymethylcellulose were tested, with glucose as positive control, and a "no carbon source well", as negative control. Growth was monitored after 5 days and results were recorded as follows: Growth equivalent to the negative control (-), growth better than negative control but not as robust as the positive control (+), growth equivalent to the positive control growth (++), growth better than the positive control (+++) (Table 3).

Taxonomical identification

Taxonomic identification was obtained by PCR amplification, sequencing and analysis of the nearly complete 16S rRNA locus using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'- TACGGYTACCTTGTTACGACTT-3'). PCR thermal cycling is described elsewhere [6]. PCR products were sequenced by Macrogen (Korea). Sequences were assembled using the CLC Main Workbench 7.0 program and compared with SILVA Living Tree Project (release109) database [7]. Sequences were aligned using SINA [8] and the phylogenetic tree was constructed using maximum parsimony DNA distance algorithm with ARB 5.5 [9].

Statistical analysis

The data obtained were analyzed with SAS 9.4 software using a factorial model 9x7 (nine microorganism and seven isolates) with Fisher's protected least significant difference (LSD) test for means comparison. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Isolation of Actinobacteria

A large number (374) of suspected Actinobacteria colonies were isolated. Pre-

treatment with CaCO₃, produced 275 isolates and application of the direct plating method yielded 99 isolates, whereas heat treatment did not produce isolates.

Antimicrobial activity

A number of 65 isolates (17.4 %) showed antibacterial activity against at least one of the bacteria tested. A significant number of isolates (149; 39.8 %) also exhibited antifungal activity to at least one of the fungus tested, showing inhibitions halos between 10 – 35 mm,

corresponding to a good or moderate activity, as shown in Figure 1. Statistical analysis showed an interaction between the two factors (Actinobacteria isolates and fungus strain), implying that each Actinobacteria isolate was most effective with a certain fungus strain.

Seven isolates displaying high antibacterial and antifungal activities were selected for further study. This group consisted of strains with at least moderate activity (++) or high activity (+++) against 1 fungal strain and 2 tested bacteria (Table 1).
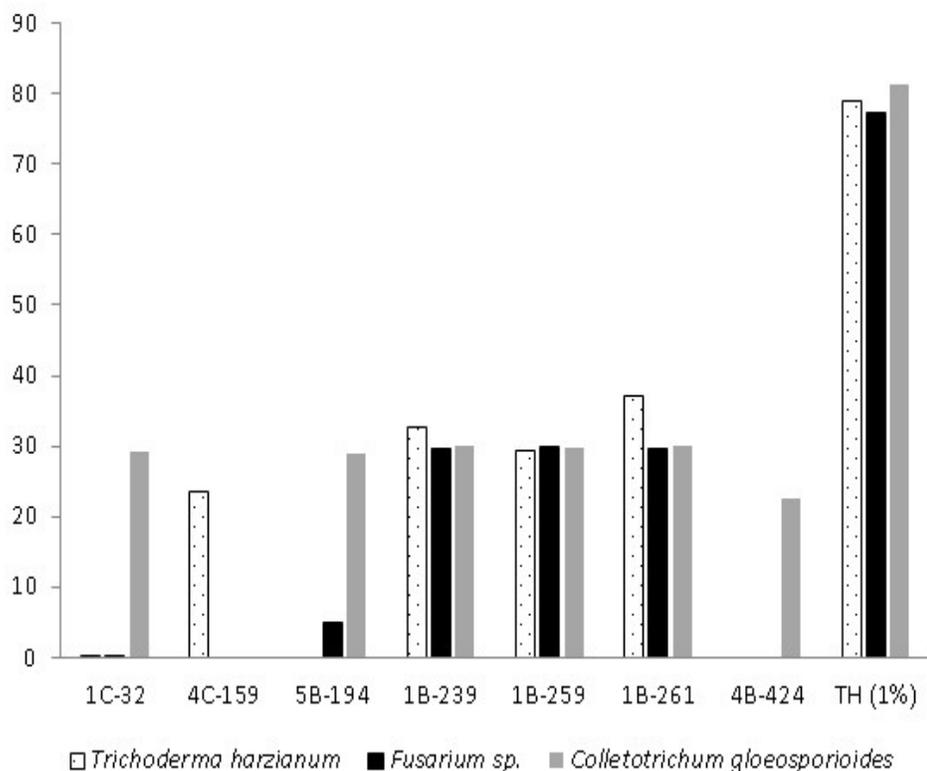

Figure 1: Diameter of inhibition halos of actinobacteria isolates with antifungal activity against *Trichoderma harzianum, Fusarium sp* and *Colletotrichum gloeosporioides*. Terbinafine hydrochloride 1% (TH) was used as control

Table 1: Antimicrobial activity of seven selected strains against fungi and bacterial strains

Isolated strain	Microorganism								
	1	2	3	4	5	6	7	8	9
1B-239	++	+	-	-	-	++	+++	+++	+
1B-259	+	++	+	++	-	+	++	++	-
1B-261	-	++	-	+	-	+	++	+++	++
1C-32	+	-	+	+	-	-	-	++	++
4B-424	-	+	-	+	-	-	-	+++	-
4C-159	+	++	++	++	-	+	-	+++	-
5B-194	-	-	+	+	-	++	+	+++	-

Microorganisms 1: E. coli. 2: S. aureus. 3: A. baumannii. 4: B. subtilis. 5: K. pneumoniae. 6: T. harzianum H5. 7: Fusarium sp. H24. 8: C. gloeosporioides 9: C. violaceum

Table 2: Morphological characteristics of isolates and closest relatives*

Isolate/closest relative		SCA	ISP1	ISP2	ISP3	ISP4	ISP5
				Medium			
1B-239	AM	W	W	W	B		
	SM	LGB	C	Br	W	YBr	LY
	RS	LBr	LY	DY	B	LY	B
S. lunalinharesii	SM	NR	NR	NR	NR	YBr	Ybr
1B-259	AM	LG	W	B	W	OG	W
	SM	W	C	W	C	W	B
	RS	LY	Y	LY	Y	Y	B
S. kasugaensis	AM	NR	NR	CGB	TW	OG	PB
1B-261	AM	W	LO	LOG	W	LBG	W
	SM	C	W	W	C	W	C
	RS	B	B	O	W	DY	B
1C-32	AM	W	YBr	W	G	GP	W
	SM	GBr	Br	GBr	C	B	DY
	RS	BY	YBr	DY	GrY	B	Y
K. putterlickiae	AM	NR	NR	DG	G	DG	DG
4B-424	AM			G	W	W	W
	SM	Y	DY	Y	Y	Br	LY
	RS	Y	DY	Y	LY	Br	LY
S. variegatus	AM	NR	NR	NR	WLP	NR	WLP
S. flavofungini	AM	NR	NR	NR	NR	WOY	NR
4C-159	AM	BR	LBr	DBr	Br	GBr	WPP
	SM	LBr	GrY	C	C	C	C
	RS	DY	O	O	Y	Gr	BY
S. griseofuscus	AM	NR	NR	Red or gray color series			
S. murinus	RS	NR	NR	YMR	GY	YMR	GY
S. costaricanus	SM	NR	NR	LY	Br	Y	LY
5B-194	AM	Br	GG	LBr	LBr	BBr	W
	SM	PG	PY	PBr	DBB	B	PR
	RS	GrBr	GrBr	Br	DY	B	BO
S. misionensis	RS	NR	NR	OBr	GY	YBr	OBr
S.phaeoluteichromatogene	AM	NR	NR	NR	Br	NR	NR

* Refer to "Key to isolates and their characteristics" subheading for nomenclature footnote

Table 3: Carbon source utilization of selected isolates

Isolate	1	2	3	4	5	6	7	8	9	10
					Carbon source					
1B-239	++	-	+++	+++	++	-	-	+	-	-
1B-259	++	-	+++	+++	++	-	-	+++	++	-
1B-261	++	+	+++	+++	+++	-	++	+++	+	-
1C-32	++	-	+	+++	-	+	-	-	++	-
4B-424	++	-	+	++	++	-	-	+	-	-
4C-159	++	+	++	+++	+++	-	++	-	+	-
5B-194	++	+++	+++	+++	+++	+	+++	+	+++	-

Carbon source 1: glucose 2: lactose 3: galactose 4: maltose 5: ribose 6: sucrose 7: xylose 8: inositol 9: fructose 10: carboxymethylcellulose Growth equivalent to the negative control (-), growth better than the negative control but not equivalent to the positive control (+), growth equivalent to the positive control growth (++), growth better like the positive control (+++)

Taxonomical identification

16S rDNA sequences were compared with SILVA Living Tree Project (release 109) database, which confirmed that all isolates belonged to Streptomycetaceae family with similarity values beyond 98 % to the closest neighbors. Maximum parsimony phylogenetic analyses is shown in Figure 2.

DISCUSSION

Enhanced recovery of *Actinobacteria* using the $CaCO_3$ pretreatment may be explained by the resulting increase in pH which promotes the growth of *Actinobacteria* propagules [10]. Several *Actinobacteria* isolation protocols, utilize heating and drying stages to select heat-resistant microorganisms capable of forming spores [11].
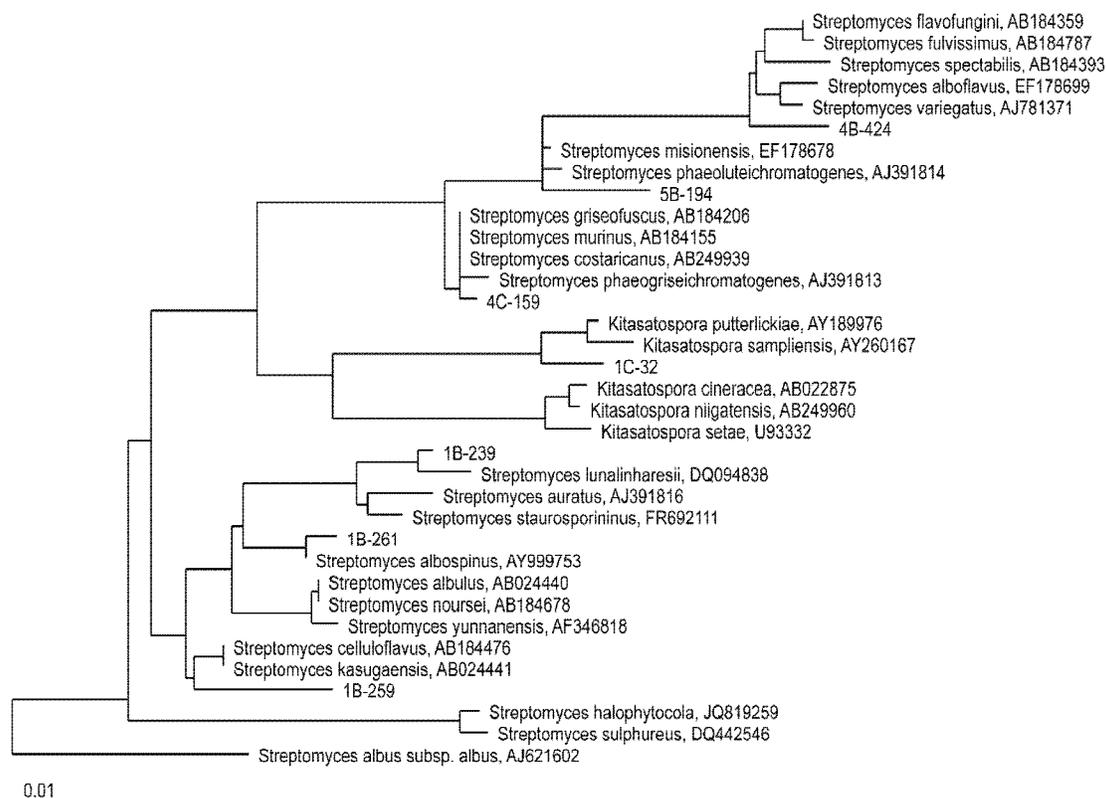
Figure 2: Phylogenetic tree of selected isolates constructed using maximum parsimony method

Williams and coworkers have suggested thermal treatments between 40-50 °C for 2-16 h for selective isolation of *Actinobacteria*, reducing significantly accompanying flora [12]. Probably this method was not successful due the temperature used compared with exposure time.

The results suggest that all 7 strains characterized in this study belong to the *Streptomycetaceae* family. Isolate 5B-194 had close similarity with two *Streptomyces* species, but according to similarities with macroscopic characteristics (table 2) and carbon source utilization it's probable that this isolate belongs to *Streptomyces misionensis* species. Isolate 4C-159 showed close similarity with three *Streptomyces* species, however doing the comparison between macroscopic characteristics and carbon source utilization was not possible to suggest which species belongs because differences were significant. Considering that most recent definition of a prokaryotic species based on 16S rDNA sequence similarity is determined by a minimum similarity of 98.7 % [24] strains 1B-259, 1C-32 and 4B-424 showed similarity levels to their closest neighbours below this value, suggesting that these isolates may represent new species from the *Streptomycetaceae* family. These strains show

some differences in terms of macroscopic characteristics and carbon source utilization respect to closest neighbours. Strains 1B-239 and 1B-261, identified as *Streptomyces lunalinharesii* and *Streptomyces albospinus*, respectively. *Streptomyces lunalinharesii* has reported activity against *Aspergillus niger, Candida albicans, Bacillus pumilus* and *Desulfovibrio alaskensis* [13,14], as well as several phytopathogenic fungi [15]. *Streptomyces albospinus* produces the fungicide phenamide against *Phaeosphaeria nodorum* [16] and two new compounds with antibacterial activity against methicilin resistant *S. aureus* (MRSA) [17].

Of all isolates obtained (374 actinobacteria isolates), a large number of them were active against *Bacillus subtilis,* consistent with previous studies, [18] and against *A. baumannii* and *K. pneumonia* which are two carbapemen-resistant microorganisms that have become problematic due to the absence of effective medication [19]. Four strains isolated in this study showed inhibition of *A. baumannii,* with two of them being able to inhibit *K. pneumoniae.* Considering the fact that *K. pneumonia* used in this study is resistant to multiple antibiotics including the carbapenem-iminipem it is likely that isolates

able to inhibit its growth are producers of new compounds. Our results also suggest a remarkable potential for antifungal agents, in particular against *C. gloeosporioides*. Fungi belonging to the genus *Colletotrichum* has been classified as disease-causing fungus affecting almost all agricultural crops in the world [20] as well as *Fusarium* spp [21]. For this reason, several actinobacterial isolates identified in this study may be potential candidates for biocontrol purposes as 43 showed inhibition of the three fungal strains tested.

Chromobacterium violaceum was included in this study to screen the strains for production of inhibitors of AHL-*quorum sensing*. According to these results, 257 isolates were able to inhibit pigment production in *C. violaceum* without altering the growth of bacteria, which represents an enormous potential, representing an alternative to the use of antibiotics and their induced resistance [22]. In this area, *Actinobacteria* are of interest due to their capacity to produce and secrete secondary metabolites that eventually can act as inhibitors of N-acyl-L-homoserine lactones (AHL), which mediate most Quorum sensing processes in Gram negative bacteria [23].

CONCLUSION

The results indicate a high abundance of culturable *Actinobacteria* in sediments from Guaviare River in Colombia, and also revealed a remarkable potential for 7 strains, selected as potential sources of antimicrobial compounds. Furthermore, three isolates showed differential phenotypic and genotypic profiles in comparison to those reported for the most closely related *Streptomyces* and *Kitasatospora* species, indicating they may represent new species. To the best of our knowledge, this is the first study of its type in the Colombian Orinoquía region and may help to identify new bacterial species with antimicrobial properties in this region.

ACKNOWLEDGEMENT

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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