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Phenotypic plasticity and characterization of *Chromobacterium* isolates from aquatic environment

Plasticidade fenotípica e caracterização de isolados de *Chromobacterium* de ambiente aquático

Plasticidad fenotípica y caracterización de aislados de *Chromobacterium* del medio acuático

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Abstract

The genus *Chromobacterium* spp. are gram-negative bacilli, which may or may not have characteristic purple pigmentation, and are mainly isolated from soil, water and infected patients. The main representative species of this genus is *C. violaceum*, responsible for a high mortality rate among those infected. The aim of this study was to genetically characterize and broadly compare phenotypic characteristics between the genus *Chromobacterium* species described in the literatureand bacterial isolates. This study is an experimental research, in which sequencing and phenotypic tests of bacterial isolates were carried out for comparison with species of the genus *Chromobacterium*. Two strains were identified, CRJL01 and CRJL02 that have characteristics of *Chromobacterium* spp species. These isolates showed high resistance to antibiotics, tolerance and resistance to metals, biochemical and physiological versatility of the CRJL01 and CRJL02 strains. In the sequencing of the 16S rRNA gene, the CRJL01 strain showed similarity with the *C. piscinae* strain. The CRJL02 strain showed similarity with the *C. subtsugae* strain. This work is the first report in 40 years of *Chromobacterium* spp. in the Brazilian Midwest – Goiás, in water samples. This isolated genus has a wide applicability for the pharmaceutical, food and cosmetic industries, due to the production of its purple/violet pigment known as violacein, and its bioprospecting is of great importance. Thus, this study is a kick-off for exploring your produced pigment.

Keywords: Antibiotic resistance; Tolerance to metals; Violacein; Pigment; Amplification of the 16S rRNA gene.

Resumo

O gênero *Chromobacterium* spp. são bacilos Gram-negativos, que podem ou não ter pigmentação roxa característica, são isolados principalmente do solo, água e pacientes infectados. A principal espécie representativa desse gênero é *C.*

violaceum, responsável por uma alta taxa de mortalidade entre os infectados. O objetivo deste estudo foi caracterizar geneticamente e comparar amplamente as características fenotípicas entre as espécies do gênero *Chromobacterium* descritas na literatura e isolados bacterianos. Este estudo é uma pesquisa experimental, no qual foram realizados sequenciamento e testes fenotípicos de isolados bacterianos para comparação com espécies do gênero *Chromobacterium*. Duas cepas foram identificadas, CRJL01 e CRJL02, que apresentam características de espécies de *Chromobacterium* spp. Esses isolados apresentaram alta resistência a antibióticos, tolerância e resistência a metais, versatilidade bioquímica e fisiológica das cepas CRJL01 e CRJL02. No sequenciamento do gene 16S rRNA, a cepa CRJL01 apresentou semelhança com a cepa *C. piscinae*. A cepa CRJL02 apresentou similaridade com a cepa *C. subtsugae*. Este trabalho é o primeiro relato em 40 anos de *Chromobacterium* spp. no Centro-Oeste brasileiro - Goiás, em amostras de água. Este gênero isolado tem ampla aplicabilidade nas indústrias farmacêutica, alimentícia e cosmética, devido à produção de seu pigmento roxo / violeta conhecido como violaceína, sendo sua bioprospecção de grande importância. Assim, este estudo é um pontapé inicial para explorar o pigmento produzido.
Palavras-chave: Resistência a antibióticos; Tolerância a metais; Violaceina; Pigmento; Amplificação do gene 16S rRNA.

Resumen
El género *Chromobacterium* spp. son bacilos gramnegativos, que pueden tener o no una pigmentación púrpura característica, y se aíslan principalmente del suelo, el agua y los pacientes infectados. La principal especie representativa de este género es *C. violaceum*, responsable de una alta tasa de mortalidad entre los infectados. El objetivo de este estudio fue caracterizar genéticamente y comparar ampliamente las características fenotípicas entre las especies del género *Chromobacterium* descritas en la literatura y aislamientos bacterianos. El presente estudio es una investigación experimental, en la que se realizaron pruebas de secuenciación y fenotípicas de aislados bacterianos para su comparación con especies del género *Chromobacterium*. Se identificaron dos cepas, CRJL01 y CRJL02 que tienen características de especies de *Chromobacterium* spp. Estos aislados mostraron alta resistencia a antibióticos, tolerancia y resistencia a metales, versatilidad bioquímica y fisiológica de las cepas CRJL01 y CRJL02. En la secuenciación del gen ARNr 16S, la cepa CRJL01 mostró similitud con la cepa *C. piscinae*. La cepa CRJL02 mostró similitud con la cepa *C. subtsugae*. Este trabajo es el primer informe en 40 años de *Chromobacterium* spp. en el Medio Oeste brasileño - Goiás, en muestras de agua. Este género aislado tiene una amplia aplicabilidad para la industria farmacéutica, alimentaria y cosmética, debido a la producción de su pigmento violeta / violeta conocido como violaceína, y su bioprospección es de gran importancia. Por lo tanto, este estudio es un punto de partida para explorar el pigmento producido.
Palabras clave: Resistencia antibiótica; Tolerancia a los metales; Violacein; Pigmento; Amplificación del gen rRNA 16S.

1. Introduction

The first species of the genus *Chromobacterium* isolated, was identified in 1880, diagnosed as *Chromobacterium violaceum* Bergonzini (1881) (Euzeby, 1998). According to the List of prokaryotic names (LPSN) the genus *Chromobacterium* belongs to the Neisseriaceae family, class Betaproteobacteria and has 14 species, being them *C. alkanivorans* (Bajaj et al., 2016), *C. amazonense* (Menezes et al., 2015), *C. aquaticum* (Young et al., 2008), *C. fluviatile* (Moss et al., 1978), *C. haemolyticum* (Han et al., 2008), *C. paludis* (Blackburn et al., 2020), *C. Phragmitis* (Blackburn et al., 2019), *C. piscinae*, *C. pseudoviolaceum* (Kämpfer et al., 2009), *C. rhizoryzae* (Zhou et al., 2016), *C. sphagni* (Blackburn et al., 2017), *C. subtsugae* (Martin et al., 2007), *C. vaccinii* (Soby et al., 2013) and *C. violaceum* (Bergonzini, 1881).

The genus *Chromobacterium* are free-living, aerobic and facultative anaerobic, mobile gram-negative bacilli, commonly found in aquatic environments and soils that have a great metabolic flexibility (Bajaj et al., 2016; Blackburn et al., 2017; Han et al., 2008; Kämpfer et al., 2009; Martin et al., 2007; Menezes et al., 2015; Moss et al., 1978; Soby et al., 2013; Young et al., 2008; Zhou et al., 2016). Bacteria of the *Chromobacterium* genus have their main characteristic for the production of purple pigments, known as violacein and deoxy-violacein (Blackburn et al., 2017). However, new non-pigmented bacterial strains were discovered (Bajaj et al., 2016; Hara-hanley et al., 2018).

Violacein is an indole derivative, purple or blue-violet in color, produced by the secondary metabolic pathway from tryptophan, in which two independent processes occur; an enzymatic process catalyzed by five proteins called VioABCDE and another process of non-enzymatic oxidative decarboxylation reactions (Durán et al., 2016; Hoshino, 2011). The structure of violacein is composed of 5-hydroxyindole, oxindole and 2-pyrrolidone units (Hoshino, 2011).

This biocompound has several biotechnological activities described in the literature, both activities with medical/pharmacological potential and also of industrial importance, such as antibacterial, antiviral, antifungal, antiparasitic, antioxidant, microbiota modulation, immunomodulatory, antipyretic, analgesic, anticancer, production of natural pigments and dyes (Durán et al., 2016; Durán & Menck, 2001; Numan et al., 2018; Pauer et al., 2018; Sen et al., 2019; Tuli et al., 2015; Venil et al., 2015).

Bacteria characterized with violet pigments of the *Chromobacterium* genus include opportunistic pathogens that can cause septicemia, highly resistant to antibiotics and with high rates of fatal infections in humans and animals, being considered an emerging pathogen (Chandler, 2019; Durán & Menck, 2001; Kothari et al., 2017; Yang & Li, 2011). The species described in the literature causing infections is *C. violaceum*, in which acquisition and/or maintenance of the pathogenicity island containing the type III secretion system (T3SS) provides the characteristic of virulence for this bacterium, thus changing from an environmental microorganism for an opportunist (Batista & Neto, 2017).

Infection by *C. violaceum* is associated with healthy and/or young people, and may have some predisposing factors such as: trauma, exposure to water or soil, or both (Yang & Li, 2011). Infections are difficult to treat, with cases of necrotizing metastatic lesions, abscesses and rapid progression to sepsis, the most common symptoms being fever and pain in the infected area (Kothari et al., 2017; Yang & Li, 2011). Reports associate *C. violaceum* with chronic granulomatous disease, indicating that this bacterium may be an indicator for this pathology (Justo & Durán, 2017; Meher-Homji et al., 2017).

In view of the biotechnological applications with industrial and medicinal potential of the pigment produced by the species of *Chromobacterium* spp. and its great importance due to its pathogenicity (Santos et al., 2018) and also the growing interest in bioprospecting new pigment-producing strains. This study aimed to characterize and identify two isolated strains suggestive of the genus *Chromobacteium* as they are purple pigment-producing strains in water samples from Ribeirão João Leite, Goiás, Brazil.

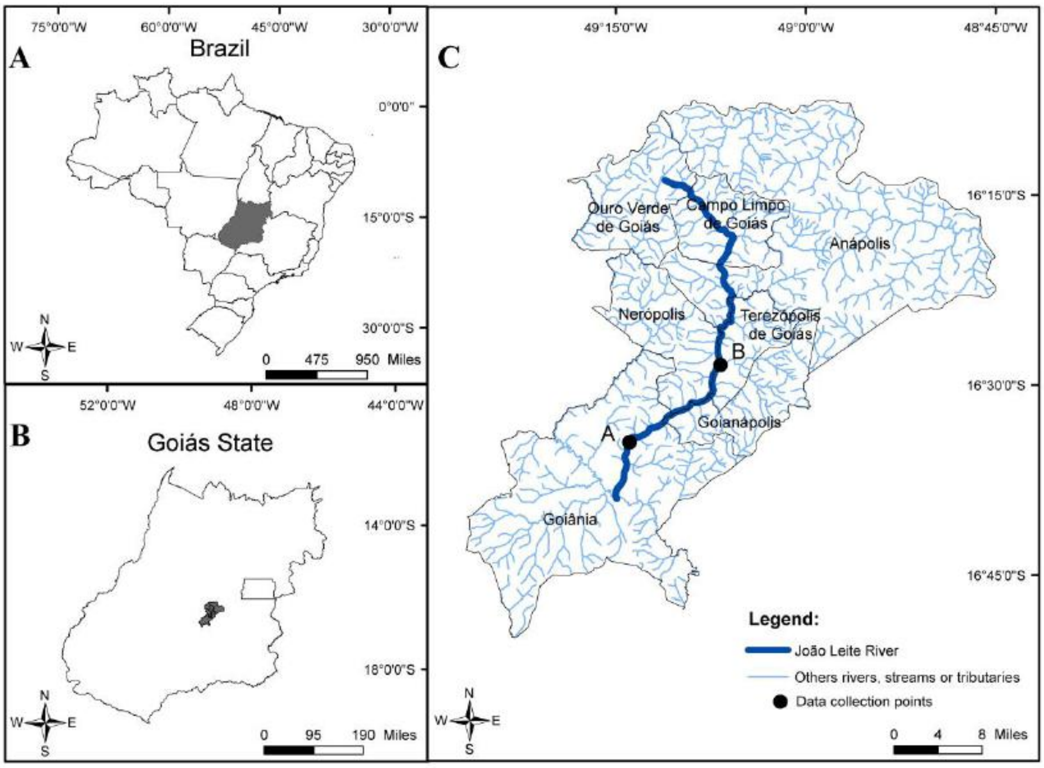
2. Methodology

2.1 Isolation

During a characterization study of fertilizing bacteria from the João Leite stream, Goiás-Brazil, bacteria were bioprospected in MacConkey and R2A media present in the water. Among the syndicates, two colonies with violet/violet morphology were observed and examined for this study.

The presence of colonies with the production of purple/violet color pigments was isolated, which belonged to points A (16°34'30.54''S; 49°13'55.02''O) e B (16°28'25.05''S; 49°6'43.87''O) described in the map of Figure 1, from which the CRJL01 and CRJL02 strains were respectively isolated.

Figure 1 - Water sample collection points from Ribeirão João Leite from which CRJL01 and CRJL02 strains were isolated.



A. Map of Brazil, highlighting the state of Goiás. **B.** Map of the state of Goiás, highlighting the basin in which Ribeirão João Leite is located, in the center of the state. **C.** Map of the cities and basin in which the Ribeirão João Leite and its tributaries pass, highlighting the course of the river and points A and B where colonies were isolated with the production of rock-colored pigments. Source: own author.

2.2 Chromosomal DNA extraction, sequencing and phylogenetic analysis

For the molecular identification of bacterial isolates, amplification of the 16S rRNA gene, sequencing and analysis of this sequence were used. For this, genomic DNA extraction was performed, following the methodology proposed by Soolingen et al. (1994), with the adaptations suggested by Oliveira et al. (2012).

For the amplification of the 16S rRNA region, the 27F primers were used (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'- AAGGAGGTGATCCAGCC-3'), described by (Weisburg et al., 1991). The polymerase chain reaction (PCR) reaction was performed with a final volume of 50 µL. 35.5 µL of miliQ water, 5 µL of sample buffer (10X), 1.5 µL of MgCl2 (50 mM), 1 µL of each primer solution (10 mM), 4 µL of dNTP solution (2.5 mM), 1µL were used of Taq polymerase (5U) and 1µL of DNA (50ng). Then, the DNA was amplified in the reaction using a thermocycler (Veriti™ 96-Well Thermal Cyclers), under the following conditions: 94 ºC for 3 minutes, 30 denaturation cycles 94 ºC for 1 minute, 55 ºC for 30 seconds and 72 ºC for 30 seconds and 72°C for 10 minutes.

To carry out the sequencing, the PCR product obtained was purified using the Agarose Extraction Kit – Cellco®, following the manufacturer's protocol. The universal primers 27F, 1541R, 926F (5′-AAACTYAAAKGAATTGACGG-3′), 530F (5'-TGACTGACTGAGTGCCAGCMGCCGCGG-3'), 519R (5'-GTNTTACNGCGGCKGCTG-3') and 907R (5'-GTNTTACNGCGGCKGCTG-3'). A reaction was performed in the ABI 3500 sequencer of the Applied Biosystems®.

The sequences obtained were analyzed according to their quality and later joined in contig, using the CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA). The homology of the contig was compared with the GenBank National Center for Biotechnology Information (NCBI) database using the nucleotide tool Basic Local Alignment

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Search Tool (BLASTn). The hits that presented the highest identities were listed as possible identifications of bacterial isolates. The sequences obtained were deposited at Genbank NCBI.

The analysis of species diversity was performed through the construction of the phylogenetic tree using the Neighbor-joining method in the MEGA X software.

2.3 Morphological, phenotypic and biochemical properties of cultures

To carry out the morphological and physiological characterization of the isolated bacterial cultures, the manual of the National Health Surveillance Agency was followed (Brasil, 2013). They were compared with other species found of the same bacterial genus identified. The comparison was made by searching the species of this genus described in the LPSN (List of Prokaryotic names with Standing in Nomenclature – https://www.bacterio.net/) together with the search for the valid publication of this species, in which all the morphological, phenotypic and biochemical information of these species, with this search being carried out in September 2021.

To test the susceptibility of these strains to antimicrobials, it was performed and interpreted using the agar fusion disk test (Kirby-Bauer antibiotic test), according to the Clinical and Laboratory for Clinical Laboratory Standards (CLSI, 2019). In which he used the Polisensidisc 15 Gram negative DME® with amikacin (AMI 30 µg), amoxicillin/clavulanic acid (AMC 30 µg), ampicillin (AMP 10 µg), aztreonam (ATM 30 µg), cefazolin (CFZ 30 µg), cefepime (CPM 30 µg), cefoxitin (CFO 30 µg), ceftazidime (CAZ 30 µg), ceftriaxone (CRO 30 µg), ciprofloxacin (CIP 05 µg), chloramphenicol (CLO 30 µg), gentamicin (GEN 10 µg), meropenem (MPM 10µg), sulfazotrim sulfamethoxazole/trimethoprim (SUT 25 µg) and tetracycline (TET 30 µg). Resistance status was assessed using the classification system that resistance to ≥3 classes of antibiotics defining multi-resistance status (MDR) (Magiorakos et al., 2012).

To test the resistance/tolerance to metals, followed the methodology proposed by Filali et al. (2000) with adaptations, performing the minimum inhibitory concentration (MIC). The metals mercury, silver, cadmium, copper, nickel, iron, zinc, barium and cobalt were tested, in which these metals were used in the forms of mercury chloride ($HgCl_2$) and silver nitrate ($AgNO_3$), cadmium chloride ($CdCl_2$), copper chloride ($CuCl_2$), nickel chlorid ($NiCl_2$), iron sulfate ($FeSO_4$), pure zinc (Zn), barium chloride ($BaCl_2$) and cobalt sulfate ($CoSO_4$).

The isolated strains were seeded in Tryptic Soy Broth (TSB) and incubated at 37 ºC for 24 hours. After growth, 100µL of cell suspensions were added, containing again 1 mL of TSB medium with different concentrations of the respective metallic elements and incubated again at 37°C for 24 hours. The concentrations in mM of each metal tested were: for $HgCl_2$ and $AgNO_3$ (12000, 6000, 3000, 1500, 750, 375, 187.5, 93.75, 46.87, 23.44, 11.72 e 5.86), $CdCl_2$, $CuCl_2$ and $NiCl_2$ (16000, 8000, 4000, 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.62 e 7.81), $FeSO_4$ and Zn (8000, 4000, 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81 e 3.91), $BaCl_2$ (1280, 640, 320, 160, 80, 40, 20, 10, 5, 2.50, 1.25, 0.62) and $CoSO_4$ (6400, 3200, 1600, 800, 400, 200, 100, 50, 25, 12.5, 6.25 e 3.12). Subsequently, it was verified by the turbidity of the medium, in which concentration tested, of the respective metals, there was bacterial growth. To confirm, 10 µl of the respective metal dilutions were added to Tryptic Soy Agar (TSA) and incubated at 37°C for 24 hours. Colony growth was indicative that the strain had a phenotypic resistance to that respective metal at that specific concentration. Thus, a minimal inhibitory concentration (MIC) was evaluated for metals.

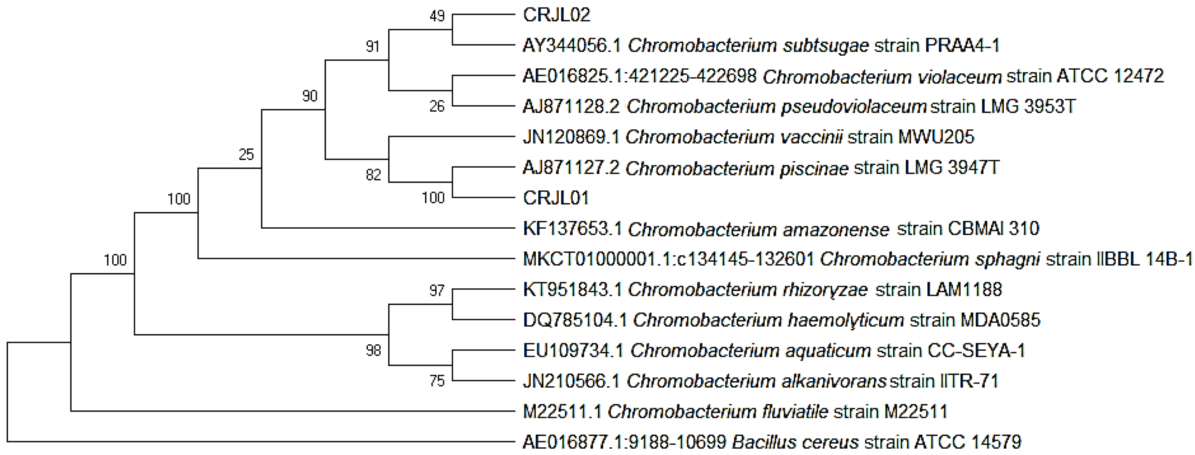
3. Results and Discussion

The 16S rRNA sequences have been deposited with the NCBI under accession number MN134084.1 for CRJL01 and accession number MN134085 for CRJL02. When compared to the database, the CRJL01 strain had the highest identity

(99.81%) with *C. Piscinae*, strain LMG 3947, sequence ID NR_114953.1. For the CRJL02 strain, it obtained the greatest identity (99.79%) with *C. violaceum*, strain ATCC 12472, sequence ID: NR_074222.1 and with *C. subtsugae*, strain PRAA4-1, sequence ID: NR_042853.1.

The multiple alignment was performed based on the sequences obtained from the 16S rRNA and the phylogenetic tree was constructed (Figure 2). The bootstrap values (rooting) 100 homology of strain CRJL01 with *C. Piscinae* strain LMG 3947 is 49% homology between strain CRJL02 and C. subtsugae, lineage PRAA4-1.

Figure 2 - Phylogenetic analysis based on 16S rRNA gene sequences.



The sequences used are available in the NCBI database and the CRJL01 and CRJL02 strains were sequenced by Sanger and then contigs were formed using the clustal W. The statistical method used was the Neighbor-joining, the phylogenetic test based on Booktstrap method with 1000 replications and the Jukes-Cantor replacement model were executed using the software package MEGA_X_10.0.5_win64_setup. Source: Own author.

Lima-Bittencourt et al. (2011), indicates that in its isolates of *Chromobacterium* spp. despite having high similarity above 97% between 16S rRNA sequences with those of *C. Piscinae*, the strains showed high genetic and phenotypic diversity, which they attributed to adaptations to the habitat (ecological barriers), suggesting the origin of new species or representing bacterial ecotypes. Thus, the data presented here indicate that the CRJL01 and CRJL02 strains are of the *Chromobacterium* genus. It can suggest that CRJL01 is a *C. piscinae* and CRJL02 is a *C. subtsugae*. However, when deposited at the NCBI, CRJL02 was described as Chromobacterium sp., as the phylogenetic analysis did not give a high value (49) to confirm the species of this strain.

A comparison was made between CRJL01 and CRJL02 strains and other species of *Chromobacterium* spp. previously described (14 species described in the LPSN). The location and characteristics of the bacterial colony were described, the data are shown in Table 1. It can be seen that most strains were isolated from environmental samples, water and soil. A comparison of the biochemical and physiological characteristics of the CRJL01 and CRJL02 strains, described in Table 2, was also performed. The data were also associated with the described characterizations of the other species of *Chromobacterium* spp. This genus are gram negative rods, the CRJL01 and CRJL02 strains are aerobic, with positive growth at 30 ºC. It has a peculiar characteristic for presenting violet coloring both in agar culture medium and in liquid medium (Figure 3).

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Table 1 - Comparisons between isolation sites and profiles of CRJL01 and CRJL02 strains with other species of the genus *Chromobacterium*.

Cepas do gênero *Chromobacterium*	**Amostras**	**Local do Isolamento**	**Perfil da colônia**	**Referências**
CRJL01	Water	João Leite stream, Goiás, Brazil	Cor violeta claro, lisa e circulares	Study
CRJL02	Water	João Leite stream, Goiás, Brazil	Violet color, smooth and circular	Study
C. alkanivorans	Soil	Industry in Eloor in the Cochin region of South India	Circular, convex, smooth and creamy-yellow pigmented	Bajaj et al. 2016
C. amazonense	Water	Negro river, Preto river da Eva municipalities in the Amazon, Brazil	Smooth and regular, violet in color	Menezes et al. 2015
C. aquaticum	Water	Yang-Ming Mountain Spring, Taipei County, Taiwan	Beige color, smooth, shiny and convex with a spreading edge	Young et al. 2008
C. fluviatile	Water and sediment	Rio Wey, Inglaterra	Round, pale violets, slightly rough	Moss, Ryall, and Logan 1978
C. haemolyticum	Patient's sputum culture	Texas, EUA.	Gray, round and raised color	Han, Han, and Segal 2008
C. paludis	Water and sediment	Langrells Island well/tank near the mouth of the Nanticoke River in Dorchester County, Maryland, EUA	Smooth, convex and deep violets with regular margins	Blackburn et al. 2020
C. phragmitis	Water	Tidal marshes of the Potomac and James Rivers in Maryland and Virginia, USA	Smooth, convex and violet colonies with regular margins	Blackburn et al. 2019
C. piscinae	Water	Lagoon in Sungai Buloh, Malaysia	Smooth, shiny and convex with expanding, violet edges	Kämpfer, Busse, and
C. pseudoviolaceum	Environment	Unreported	Smooth, shiny and convex with expanding, violet edges	Scholz 2009
C. rhizoryzae	Rice roots (*oryzasativa*)	Hubei Province, China	Beige and plain color	Zhou et al. 2016
C. sphagni	Water	Sphagnum West Virginia and Maine, EUA	Smooth, convex and violet with regular margins	Blackburn et al. 2017
C. subtsugae	Soil	Mountain Region Catoctin, Maryland, , EUA	Cream colored but become deep violets starting from the center	Martin et al. 2007
C. vaccinii	Wild cranberry soil	Truro, MA, USA	Round convex, smooth and shiny, starting off as cream and quickly turning dark purple from the center of the colony	Soby et al. 2013

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Table 2 - Comparisons of biochemical and phenotypic properties between CRJL01 and CRJL02 strains with other species of the genus *Chromobacterium*.

Biochemical and Physiological Characteristics	**Genus strains *Chromobacterium***													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Respiration	aeróbio	aeróbio	aeróbio	aeróbio	aeróbio	aeróbio	aeróbio	aeróbio facultativo	aeróbio facultativo	aeróbio	aeróbio	aeróbio facultativo	aeróbio	aeró
Assimilation														
Malic acid	-	-	ND	+	+	ND	+	+	+	ND	ND	+	-	ND
Arabinose	+	+	+	-	-	+	+	-	+	ND	ND	+	-	ND
Trisodium citrate (Citrate)	+	+	+	+	+	+	+	-	+	-	-	+	-	+
Glucose	+	+	+	+	+	+	+	+	+	ND	ND	+	+	+
Lactose	+	+	ND	ND	ND	+	ND	-	-	ND	ND	+	ND	ND
Maltose	+	+	-	-	-	ND	-	+	+	ND	ND	+	ND	ND
Mannitol	+	+	ND	-	-	+	+	-	+	-	-	+	-	ND
Sucrose	+	+	ND	ND	ND	+	ND	ND	ND	ND	ND	+	ND	+
Fermentation														
Glucose	+	+	+	+	+	+	+	+	+	+	+	ND	-	+
Lactose	-	-	-	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	ND
Sucrose	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Growth														
with NaCl 2%	-	-	+	+	+	ND	ND	-	+	+	+	+	+	+
with NaCl 3%	-	-	-	+	+	ND	ND	-	ND	+	+	+	-	-
with NaCl 3,5%	-	-	-	-	+	ND	ND	-	ND	+	+	+	ND	ND
with NaCl 5%	-	-	-	-	-	ND	ND	-	ND	ND	ND	-	ND	ND
with NaCl 6,5%	-	-	-			ND	ND	-	ND	ND	ND	-	ND	ND
with ph 10	-	-	+	+	+	ND	ND	-	-	-	-	+	ND	-
with ph 5	+	+	+	-	-	ND	ND	+	ND	+	+	+	+	+
in MacConkey agar medium	+	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+	ND	ND
in salted Mannitol agar medium	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Production of														
Acetoin	+	+	ND	ND	-	-	ND	ND	ND	ND	ND	+	ND	ND
Arginine dihydrolase	+	+	+	+	+	-	+	+	+	ND	ND	+	+	ND
Catalase	+	+	+	-	-	+	+ (fraco)	ND	ND	-	-	+	ND	ND
Coagulase	+	+	ND	ND	ND	ND	ND	+	+	ND	ND	ND	ND	ND

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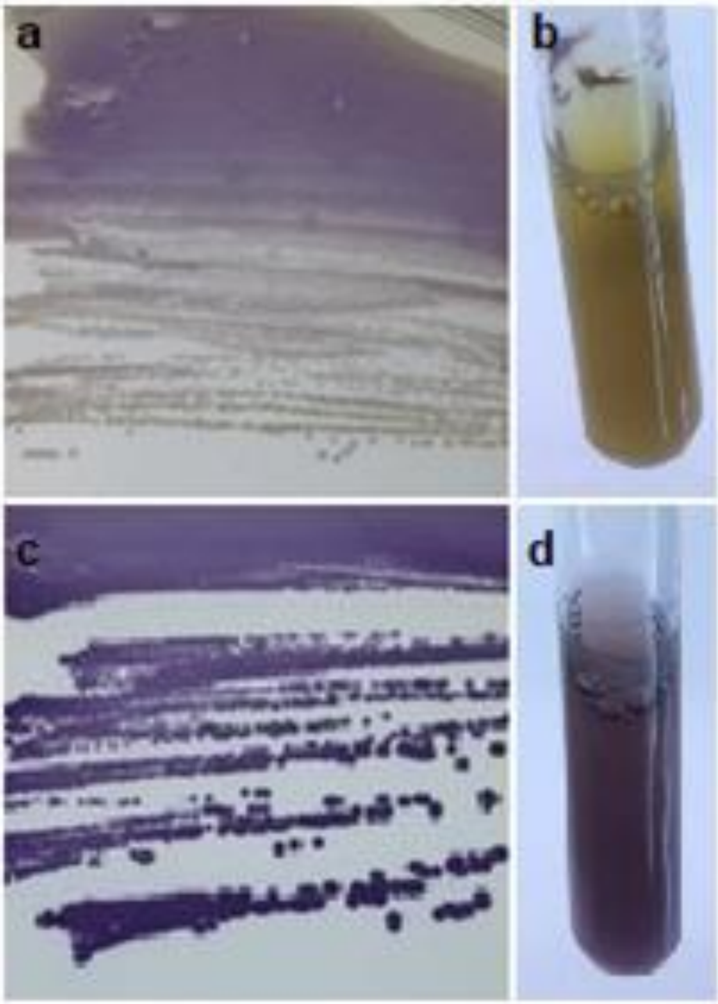
Phenylalanine desaminase	-	-	ND	ND	ND	-	ND	ND	ND	ND	ND	+	ND	ND
H_2S	-	-	-	ND	-	-	ND	ND	ND	ND	ND	-	ND	ND
Oxidase	+	+	ND	+	+	ND	+	ND	ND	+	+	-	ND	ND
Urease	-	-	+	-	-	-	-	-	+	ND	ND	-	-	ND
Hydrolysis of														
Esculine	-	-	ND	+	+	-	-	-	-	-	-	ND	-	-
Gelatin	+	+	+	+	+	+	+	+	ND	ND	ND	+	ND	ND
Tryptophan to indole conversion	-	-	ND	ND	-	-	-	-	-	ND	ND	+	-	ND
Lysine decarboxylation	-	-	ND	ND	-	-	ND	ND	ND	ND	ND	ND	ND	ND
Hemolysis	A	A	ND	ND	+	A	β	+	+	ND	ND	+	ND	-
Motility	+	-	+	+	+	+	ND	+	+	+	+	+	ND	ND
Nitrate Reduction	+	-	-	-	+	+	+	+	+	+	+	+	+	+

1. Cepa CRJL01; 2. CRJL02; 3. *C. alkanivorans* (Bajaj et al., 2016); 4. *C. amazonense* (Menezes et al., 2015); 5. *C. aquaticum* (Young et al., 2008); 6. *C. fluviatile* (Moss et al., 1978); 7. *C. haemolyticum* (Han et al., 2008); 8.*C. paludis* (Blackburn et al., 2020); 9. *C. phragmitis* (Blackburn et al., 2019); 10. *C. piscinae*, 11. *C. pseudoviolaceum* (Kämpfer et al., 2009); 12. *C. rhizoryzae* (Zhou et al., 2016); 13. *C. sphagni* (Blackburn et al., 2017); 14. *C. subtsugae* (Martin et al., 2007); 15. *C. vaccinii* (Soby et al., 2013); 16. *C. violaceum* (Bergonzini, 1881); $NaCl_2$: sodium chloride; ND; Not detected in reference consulted. +: Positive result. -: negative result; H_2S: hydrogen sulfide. Source: Own author.

Figure 3 - Microscopic aspect of the colony of CRJL01 and CRJL02 strains.



All figures were obtained with 24 hours of growth. **a.** CRJL01 strain on R2A agar medium; **b.** CRJL01 strain in LB broth medium; **c.** CRJL02 strain on R2A agar medium; **d.** CRJL02 strain in LB broth medium. Source: Own author.

The phenotypic differences between the strains of *Chromobacterium* spp. it may be due to the existing genetic diversity. Which corroborates the data from Lima-Bittencourt et al. 2007, that when analyzing the *Chromobacterium* spp. from three Brazilian environments, obtained high biochemical versatility among the isolates. Dall'Agnol et al. 2008, also demonstrated a high genetic and phenotypic diversity of C. violaceum isolated from aquatic environments in the state of Pará, Brazil.

Isolates CRJL01 and CRJL02 have biochemical versatility due to being environmental isolates, growing in high variation and competitiveness of the environment. Many microorganisms use phenotypic variation for survival in the environment (Balaban et al., 2004; Guantes et al., 2016; Zimmermann et al., 2015). Despite the high values of identity and homology between the CRJL01 strain and the *C. piscinae* strain LMG 3947, some differences were detected, such maltose and citrate assimilation, catalase enzyme production and growth with $NaCl_2$ (Table 2). The differences found between strain CRJL02 and *C. subtsugae*, strain DSM 17043 or PRAA4-1 were growth with 2% $NaCl_2$, hemolysis pattern and reduction of nitrate to nitrite (Table 2).

In the Brazilian Midwest (Goiás), the first report of isolation of Chromobacterium spp. was described by Reis et al. (1972), isolated from the waters of the Ribeirao Dois Irmãos in Goiânia, Goiás, Brazil. In subsequent years, Da Freitas et al. (1974), isolated in water samples from three municipalities in the state of Goiás (Hidrolândia, Ipameri and Cavalcante).

Subsequently, there was a last report of Rodrigues (1979), in water from slaughterhouses in the municipalities of Catalão and Luziânia, assigning a new species, called *C. goianiensis*. This described species obtained in its biochemical tests growth in macConkey médium agar, hemolysis, did not produce hydrogen sulfide (H_2S), fermented only glucose, catalase, urease and oxidase positive and conversion of tryptophan to indole, production of phenylalanine deaminase and negative citrate assimilation, differing from CRJL01 and CRJL02 isolates (Table 2) only in the biochemical tests of citrate assimilation and urease production. Thus, the CRJL01 and CRJL02 strains can be an indication of being *C. goianiensis*. However, this species has not been genetically described for comparison.

The CRJL01 strain was resistant to the antibacterials ceftazidime, ampicillin, ceftriaxone, cefazolin, gentamicin, chloramphenicol, amoxicillin plus clavulanic acid, amikacin, cefepime, aztreonam and cefoxitin, it had intermediate resistance to sulfazotrim and sensitivity only to tetracycline and ciprof. The CRJL02 strain was resistant to the antibacterials ceftazidime, ampicillin, ceftriaxone, cefazolin, gentamicin, chloramphenicol, amoxicillin plus clavulanic acid, cefepime, aztreonam and cefoxitin and sensitivity to sulfazotrim, amikacin, tetracillin and ciprofloxacin. The isolates from this study demonstrated MDR to the antibacterials tested.

Similar results have been demonstrated by Newaj-Fyzul et al. (2008), who performed a bacterial isolation study in water samples from three farm lakes in eastern and central Trinidad, within the study, the frequency of resistance of 12 *Chromobacterium* spp. among these isolates, resistance against ampicillin, oxytetracycline and erythromycin was found. In the study of Ravi et al. (2019), a *Chromobacterium* spp. demonstrated resistance to six antibiotics out of fifteen antibiotics tested, also demonstrating MDR. In the study of Lima-Bittencourt et al. (2011) isolates of *Chromobacterium* spp. demonstrated high resistance to β-lactams.

In the study of Freitas et al. (2019), which isolated a strain of the genus *Chromobacterium* spp. in an Amazon lake, in Brazil, indicated that this strain has resistance to carbapenems. Another study of Gudeta et al. (2016), indicated that the genus *Chromobacterium* spp. have high identity and were phylogenetically related to KPC (class A carbapenemase) suggesting that this bacterial genus may have played a role in the evolution of KPC.

Reports of *Chromobacterium* spp. resistant must be careful. Although the infection is rare in humans, its evolution is rapid, causing abscess in vital organs, with high mortality due to high resistance to antibiotics. The genera related to infections mentioned in the literature are *C. violaceum* (Alves De Brito et al., 2004; Anuradha et al., 2018; Madi et al., 2015; Martinez et al., 2000; Zala et al., 2018) and C. *haemolyticum* (Han et al., 2008; Miki & Okada, 2014; Okada et al., 2013). However, there is no study of other species of *Chromobacterium* described, they have pathogenicity. Plus its high resistance in the environment can serve as a reservoir and dissemination of antibiotic resistance genes (Gudeta et al., 2016).

The MIC results for the metals against the strains (CRJL01 and CRJL02) were resistant to the highest tested concentrations of $BaCl_2$(2560 µM), $CoSo_4$(12800 µM), $FeSO_4$ (8000 µM) and Zn (8000µM), the CRJL01 strain was resistant to concentrations of $CdCl_2$(4000 µM), $CuCl_2$(4000 µM), $HgCl_2$(1500 µM), $NiCl_2$(8000 µM) and $AgNO_3$(93.75 µM). The CRJL02 strain was resistant to concentrations of $CdCl_2$(8000 µM), $CuCl_2$(250 µM), $HgCl_2$(375 µM), $NiCl_2$(16000 µM) and $AgNO_3$(93.75 µM).

When performing the MIC test for metals, it was observed that barium, cobalt, iron, mercury and zinc, in high concentrations, partially or totally inhibited the production of purple pigment of the CRJL01 and CRJL02 strains. New tests must be carried out to verify if there is any relationship between metal concentrations and pigment production. It is known that the production of violacein is controlled by the quorum sensing (de Oca-Mejía et al., 2014) and a study suggests that cadmium disrupts quorum-sensing-related proteins (Newaj-Fyzul et al., 2008; Thornhill et al., 2017). Suggesting that stress caused by metals may inhibit violacein production. However, for proof, additional studies must be carried out.

The strain *C. pseudoviolaceum* GCC-SO4, showed tolerance to metals cadmium, lead, iron and copper and resistance to antibiotics methicillin and penicillin (Nath et al., 2019). Under alkaline conditions, cyanogenic bacteria, including *C. violaceum*, had the ability to leach the metals copper, iron, silver, gold and zinc; exhibiting maximum bioliability compared to the other bacteria tested in the study (Pradhan & Kumar, 2012). *C. violaceum* is one of the most cited and studied bacterial species with potential for metal bioremediation (de Alencar et al., 2017). In this way, the resistance and tolerance to the metals tested here, the biotechnological potential for metal degradation of the CRJL01 and CRJL02 strains can be explored.

4. Conclusion

Considering the data presented here, this study is an indication that *Chromobacterium* spp. are circulating in the aquatic environment of central western Brazil – Goiás. Being the first report of *Chromobacterium* spp. in the last 40 years, in water samples, showing MDR to the tested antimicrobials, also a high resistance and tolerance to metals and a great phenotypic and biochemical diversity. The report of this study is of paramount importance, given the great potential for infection and mortality that this bacterial genus can induce.

The sample isolation site is a river, which serves to supply the population of the state of Goiás and is also used in general for recreation and leisure activities, for water collection by industries and agriculture. Thus, the population has had direct contact with this isolation habitat of the CRJL01 and CRJL02 strains.

Another important point is the biotechnological potential that the strains have and studies are being carried out on the production of pigment and the use of the strain for bioremediation of metals. In-depth studies should be taken into consideration for future work on the circulation of bacterial genus in the state of Goiás and its potential in bioremediation and application of the pigment violacein produced, which has great application in the pharmaceutical, cosmetic and food industries.

Our study group is investigating the biotechnological potential of these strains, and future studies should be published soon.

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