Genetic variability in isolates of *Chromobacterium violaceum* from pulmonary secretion, water, and soil

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ABSTRACT. *Chromobacterium violaceum* is a free-living Gram-negative bacillus usually found in the water and soil in tropical regions, which causes infections in humans. Chromobacteriosis is characterized by rapid dissemination and high mortality. The aim of this study was to detect the genetic variability among *C. violaceum* type strain ATCC 12472, and seven isolates from the environment and one from a pulmonary secretion from a chromobacteriosis patient from Ilhéus, Bahia. The molecular characterization of all samples was performed by polymerase chain reaction (PCR) sequencing and 16S rDNA analysis. Primers specific for two ATCC 12472 pathogenicity genes, *hil*A and *ysc*D, as well as random amplified polymorphic DNA (RAPD), were used for PCR amplification and comparative sequencing of the products. For a more specific approach, the PCR products of 16S rDNA were digested with restriction enzymes. Seven of the samples, including type-strain ATCC 12472, were amplified by the *hil*A primers; these were subsequently sequenced. Gene *ysc*D was amplified only in type-strain ATCC 12472. *Msp*I and *Alu*I digestion revealed 16S rDNA polymorphisms. This data allowed the generation of a dendogram for

each analysis. The isolates of *C. violaceum* have variability in random genomic regions demonstrated by RAPD. Also, these isolates have variability in pathogenicity genes, as demonstrated by sequencing and restriction enzyme digestion.

Key words: 16S rDNA; Pathogenicity island; Polymerase chain reaction; Polymorphism

INTRODUCTION

Chromobacterium violaceum is a Gram-negative bacillus usually found in the water and soil in tropical regions, especially in the Negro river and its banks in the Brazilian Amazon (Hungria et al., 2005). The colonies of *C. violaceum* are purple colored, due to a colored pigment produced by the bacteria under aerobic conditions. However, some non-pigmented strains also exist in nature (Andrighetti-Fröhner et al, 2003; Antônio and Creczynski-Pasa, 2004). *C. violaceum* was first described by Boisbaudran in 1882 (Duran et al., 2001). This microorganism was first encountered in Brazil almost a century later, in 1976 (Hungria et al., 2005).

One of its most notable characteristics is the production of the purple pigment, insoluble in water and soluble in ethanol, known as violacein. Violacein has been suspected to protect the bacteria against solar irradiation, which led to considerable research into the therapeutic, antibiotic, pharmacological, and biotechnological properties of this pigment (Duran et al., 2001; Andrighetti-Fröhner et al., 2003; Antônio and Creczynski-Pasa, 2004; Carepo et al., 2004; Hungria et al., 2005).

C. violaceum is a free-living organism that usually behaves as a saprophyte. Sporadically, it becomes an aggressive human pathogen, although infections in animals are more common (Martinez et al., 2000; Chen et al., 2003; Dias et al., 2005; de Siqueira et al., 2005; Sirinavin et al., 2005; Teoh et al., 2006; Chang et al., 2007). Its pathogenic potential was first described in bulls; in humans, it was first reported in Malaysia (Lee et al., 1999; Chang et al., 2007). Recently, open reading frames (ORFs) coding for TTSS components (type III secretory system) that are strictly involved with pathogenesis were revealed by genome sequencing (Brazilian National Genome Project Consortium, 2003; Brito et al., 2004).

Although this bacteria has a low infection frequency in humans, chromobacteriosis is characterized by rapid dissemination, high mortality (Sirinavin et al., 2005), and resistance to a large number of antibiotics. ORFs associated with beta-lactam and multidrug resistance genes were also identified in its genome (Fantinatti-Garboggini et al., 2004).

A previous study has reported a case of chromobacteriosis that occurred in three brothers after recreational exposure in stagnant water in the city of Ilhéus (Bahia, Brazil); the patients developed septicemia, and purple colonies, identified as *C. violaceum*, were obtained from the tracheal aspirate, as well as the lake and its banks (Dias et al. 2005; de Siqueira et al., 2005). In this study, these isolates were analyzed under the scope of genetic diversity.

MATERIAL AND METHODS

Strains and samples

The aim of this study was to detect the genetic variability between different isolates

of *C. violaceum* by PCR sequencing of pathogenicity genes and 16S rDNA analysis. Nine strains: one type-strain ATCC 12472, one from a pulmonary secretion, two from water, and five from soil were isolated. They were numbered as follows: 1 - ATCC 12472 (type-strain); 2 - sample number 32827 (water); 3 - 43349 (water); 4 - 31620 (pulmonary secretion); 5 - 32829 (soil); 6 - 32834 (soil); 7 - 32840 (soil); 8 - 32847 (soil); and 9 - 32849 (soil). Bacterial stock cultures were stored in 20% glycerol broth at -80°C.

DNA extraction and preparation

The strains were inoculated in 3 mL nutrient broth and incubated at 36°C overnight. Subsequently, the cultures were centrifuged in a miniSpin Eppendorf (Eppendorf, Hamburg, Germany) at 9660 *g* for 10 min. The supernatant was discarded and the pellets washed with 600 µL (0.5 M; pH 8.0) EDTA. Then, 360 µL Tris-HCl (20 mM), pH 8.0, and 20 µL 25% sodium dodecyl sulfate (SDS) were added to the pellet and homogenized. The samples were incubated at 60°C for 10 min; 200 µL of 5 M NaCl was added to this mixture and gently homogenized. The tubes were cooled on ice, centrifuged at 12,000 rpm for 10 min, and processed with phenol-chloroform-isoamyl alcohol (25:24:1) to eliminate all protein residues. The DNA was then precipitated using 500 µL cold isopropanol and 20 µL 0.2 M NaCl. The resulting pellets were washed with 100 µL ethanol (70%), suspended in 50 µL Tris-EDTA, and treated with RNAse (10 µg/mL) at 37°C for 30 min.

Polymerase chain reaction (PCR) sequencing and RAPD

PCR was performed using the forward and reverse primers for *hil*A and *ysc*D genes; the primers were designed based on the complete genomic sequence of the bacteria and was available at the Virtual Institute of Genomic Research, Brazilian Genome (http://www.brgene. lncc.br/cviolaceum). The primer sequences are as follows: *hil*A, (763 bp): forward 5'-GCG AAC TGG TGA GCA AGG A-3' and reverse 5'-AGA TAG CAT TCG GCC AGG C-3'; *ysc*D (587 bp): forward 5'-GTT GGG GCA GGC GAT AGA-3' and reverse 5'-TAA CCA CAC GCC AGC CAT-3'. The sequence of 60 RAPD primers belongs to kit A, kit B and kit C kits (http://www.operon.com).

The same reaction components and cycles were used for sequencing with primers *hil*A and *ysc*D, as well as the RAPD primers. PCR amplification was performed in a 25-µL mixture, containing 2.5 µL 10X buffer, 1.0 µL MgCl$_2$ (50 mmol.L^{-1}), 2.0 µL dNTP mix (2.5 mM), 1.0 µL primer (5 pmol), 1 U Taq DNA polymerase, and 2 µL target DNA (0.5 ng/µL). The PCR program was set in the thermal cycler as follows: initial denaturation at 94°C for 4 min, 40 cycles at 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. The amplified fragments were electrophoresed on a 1.2% agarose gel, stained with ethidium bromide, and the fragments visualized on a UV transilluminator. The amplified DNA with primers *hil*A and *ysc*D was treated with exonuclease I and shrimp alkaline phosphatase, and sequenced in MegaBace 1000. The sequences were analyzed in CAP3 (http://pbil.univ-lyon1.fr/cap3.php), ClustalW (http://www.ebi.ac.uk/clustalw), and MEGA (http://megasoftware.net).

In vitro restriction profile of amplified 16S rDNA

Universal primers designed for bacteria fD1 and rD1 were used to amplify the 16S

rDNA of the samples (Weisburg et al., 1991). The D1-D2 region of large subunit ribosomal gene was used here because it usually has a higher genetic diversity than other rDNA regions. The samples were amplified in a 25 µL reaction mixture containing 2.5 µL 10X buffer, 1.0 µL MgCl$_2$ (50 mM), 2.0 µL dNTP mix (2.5 mM), 1.0 µL primer (5 pmol), 1U Taq DNA polymerase, and 2 µL DNA (0.5 ng/µL). The program was set in the thermal cycler as follows: an initial denaturation at 94°C for 4 min, 40 cycles at 94°C for 0.5 min, 54°C for 0.5 min, and 72°C for 1.5 min, and a final extension at 72°C for 7 min. The amplified fragments were treated with *Eco*RI, *Xho*I, *Bam*HI, *Hin*dIII, *Msp*I, *Kpn*I, *Alu*I, and *Hha*I. PCR fragments were separated by electrophoresing on a 1.2% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

Clustering analysis

RAPD and polymorphic bands from restriction profiles were analyzed by PAST program (http://folk.uio.no/ohammer/past) using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), based on the Dice similarity coefficient.

RESULTS

Sixty RAPD primers generated 134 polymorphic bands, representing an average of 2.23 polymorphic bands for each primer. Data analysis indicated a wide diversity among the isolates (Figure 1). The main group of bands was generated by the soil strains 5, 8, and 9, which showed a similarity ranging from 0.70 to 0.81. The soil strain 7 presented intermediate similarity with this group (0.55), while the other strains showed low genetic similarity (0.25 to 0.41). Type-strain ATCC 12472, the only one in this study not isolated from Ilhéus, BA, but from Malaysia, showed an average similarity of 0.37 with all isolates (0.33 to 0.41). The strains obtained from the pulmonary secretion showed an average similarity of 0.39 with the other strains (0.34 to 0.41). Therefore, the isolates from water (2 and 3), pulmonary secretion (4), and Malaysia (1) are genetically more diverse than the strains isolated from the soil (5, 7, 8, and 9).
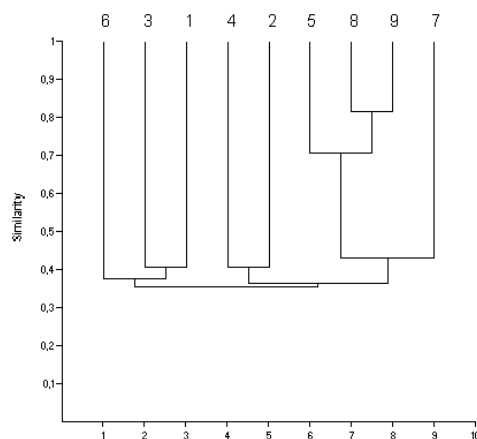

Figure 1. Dendogram designed by UPGMA method, based on the Dice similarity coefficient, from RAPD data. 1: ATCC 12472; 2 and 3: isolates from water; 4: isolate from pulmonary secretion; 5 to 9: isolates from soil.

All strains excluding 2 and 5 generated a 763-bp PCR product when sequenced with *hil*A primers (Figure 2). The analyzed strains (1, 3, 4, 6, 7, 8, and 9) generated sequences of good quality from the *hil*A gene. Based on the alignment of the seven strains, a group without geographic correspondence was identified (Figure 3). Three of the soil strains (6, 8, and 9) were very similar, and the other (7) was the most divergent among all strains. One strain isolated from water (3) was similar to this main group. Conversely, the strain isolated from a pulmonary secretion (4) and the type-strain (1) did not form any group. Here, as observed in RAPD analysis, the two isolates were less similar.
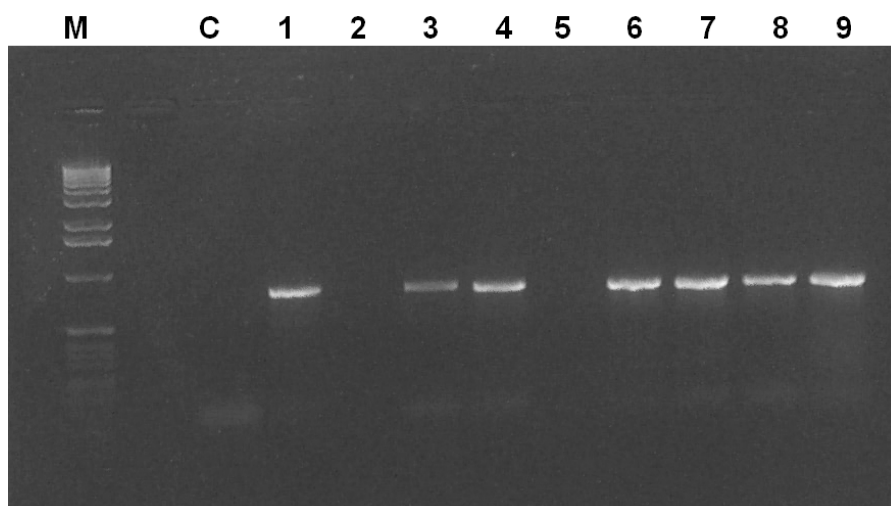

Figure 2. PCR of *hil*A gene. *Lane M* = ladder (1 kb); *lane C* = negative control; *lane 1* = ATCC 12472; *lane 2 and 3* = isolates from water; *lane 4* = isolate from pulmonary secretion; *lane 5* to *9* = isolates from soil. Strains 2 and 5 showed no amplified fragments.
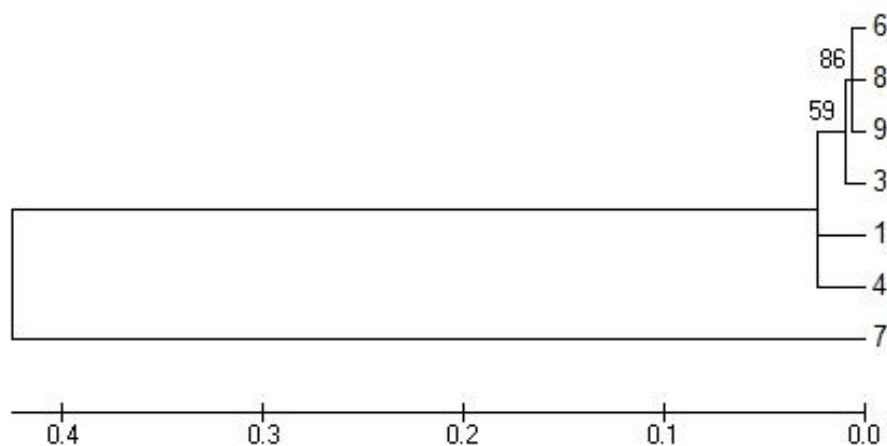

Figure 3. Linearized tree designed by neighbor joining method based on Clustal W multiple alignment of *hil*A gene sequences, showing dissimilarity data. Bootstrap 10,000X. 1: ATCC 12472; 3: isolates from water; 4: isolate from pulmonary secretion; 6 to 9: isolates from soil.

The fragment amplified from primers fD1 and rD1 were of the expected size (1500 bp). The 16S rDNA did not express restriction sites for enzymes *Hin*dIII, *Bam*HI, and *Kpn*I; however, the sequence showed one restriction site each for enzymes *Eco*RI, *Xho*I, and *Hha*I. Polymorphisms were detected only when enzymes *Msp*I and *Alu*I (Figure 4) were used. This result is consistent with the characteristic of *Hha*I, *Msp*I, and *Alu*I, which recognize four bases.

16S rDNA analysis grouped the strains 5, 8, and 9 together, with a similarity of 100% (Figure 5). This was consistent with the characteristic of this conserved region in bacterial species.
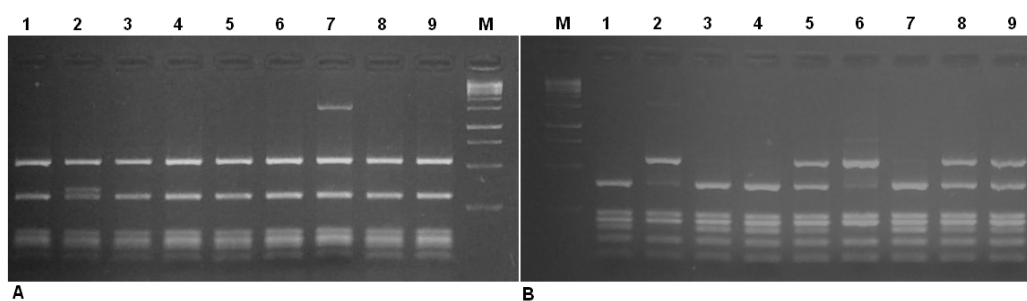

Figure 4. Digestion of amplification products of 16S rDNA extracted from different strains of *Chromobacterium violaceum* (*lane 1* = ATCC 12472; *lanes 2* and *3* = isolates from water; *lane 4* = isolate from pulmonary secretion; *lane 5* to *9* = isolates from soil); the amplification was performed with different restrictions enzymes: A - *Msp*I; B - *Alu*I; *lane M*: ladder (1 kb).
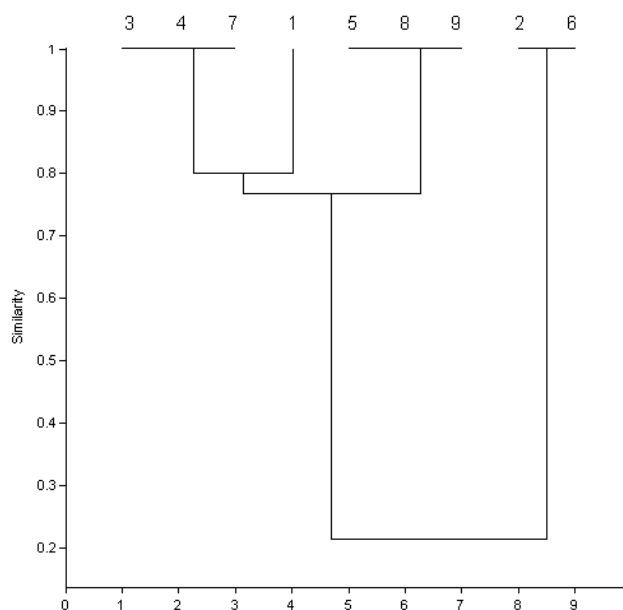

Figure 5. Dendogram designed by UPGMA method, based on Dice similarity coefficient, of 16S rDNA restriction polymorphism data. *Lane 1* = ATCC 12472; *lanes 2* and *3* = isolates from water; *lane 4* = isolate from pulmonary secretion; *lane 5* to *9* = isolates from soil.

DISCUSSION

RAPD data identified different genetic groups in this study. RAPD has been previously used to identify the different serotypes of pleuropneumonia-causing pathogens in pigs (*Actinobacillus pleuropneumoniae*) when the usual methods proved to be ineffective. Epidemiologic studies must aim to analyze the distribution of a sample within an area (Costa et al., 2004). Although not many groups reflected these two geographic regions, the isolates from Malaysia and from the pulmonary secretion demonstrated the low similarity among the strains.

Polymorphisms in pathogenicity and virulence factor genes have been subjected to widespread molecular analysis (Nightingale et al., 2005; González-Escalona et al., 2008; Rossi et al., 2013). In this study, the type-strain ATCC 12472 generated a 587-bp PCR fragment, as expected, for the *yscD* gene. This PCR product was sequenced, and BLAST analysis showed 100% similarity between our sequence and the sequence available in GenBank. The fact that the other strains did not produce this fragment is not enough to argue that the *yscD* gene is absent. Therefore, the possibility of a mutation at the primer annealing sites must be considered; a Southern blot would clarify this question, as well as determine the number of copies of the gene, if present.

Southern blot analysis was used to confirm the presence or absence of the *hil*A gene in strains 2 and 5. The primers used to amplify these two pathogenicity genes were designed based on type strain ATCC 12472; therefore, this isolate was used as the positive control. All analyzed strains in another study of pathogenicity genes gave rise to amplified fragments in a multiplex PCR (Scholz et al., 2006). Conversely, some key virulence genes present in the ATCC 12474 type-strain were absent in the strains isolated from black water in the Brazilian Amazon (Hungria et al., 2005).

Although both RAPD and sequencing analysis indicate the higher similarity between strains 8 and 9 (both from soil), the sequence analysis also added strain 6 to this group, which was not obtained with RAPD analysis. These genes belong to the pathogenicity islands and are susceptible to horizontal transmission, differing from the whole genome. On the other hand, isolates 1, 4, and 7 were congruently less similar when analyzed by both methods. Only strain 6 was not congruent among the dendograms obtained from RAPD and sequencing analysis.

It was observed that, while the RAPD data of the strains showed similarity ranging from 0.25 to 0.81, the similarity among the sequencing data of all strains was > 0.99. *C. violaceum* genome contains 12 insertion sequences (IS) and ORFs similar to many free-living species, which are correlated with its interaction with the environment (de Almeida et al., 2004). Considering the high plasticity of this microorganism, caused specifically by the lateral gene transfer (de Almeida et al., 2004), the high degree of polymorphism in the whole genome detected by RAPD markers may reflect the typical diversity of *C. violaceum*, while the low polymorphism of the *hil*A gene may reflect a housekeeping gene shared with other taxonomic groups.

The primers fD1 and rD1 have been successfully used in closely related Proteobacteria, such as *Neisseria* and *Pseudomonas*, under the scope of genetic diversity (Weisburg et al., 1991). In addition to the difference in the similarity percentage, our results from 16S rDNA analysis are congruent with RAPD data. Even a highly conserved gene could be used to differentiate strains 5, 8, and 9 from 1 and 4, and 1 and 4 from each other. 16S rDNA analysis has been widely used for phylogenetic and evolutionary studies because of its high specificity and conservation among different taxonomic groups (Weisburg et al., 1991; Aakra et al., 1999; Liu et al., 1999; Drancourt et al., 2000; Hungria et al., 2005). Additional polymorphisms in the

16S rDNA could be detected using other enzymes or by specific sequencing of the fragment.

Restriction analysis could also be performed in a housekeeping gene. In fact, it has been previously demonstrated that the *rec*A gene can be used as a PCR target gene to detect *C. violaceum*. Digestion with restriction enzymes allows us to differentiate between different isolates, and separate them into three distinct genospecies (Scholz et al., 2005).

In this study, the wide genetic diversity among nine strains of *C. violaceum* was demonstrated; the use of RAPD markers resulted in a greater diversity, compared to sequencing of the conserved genes. Moreover, the two analyses were partially congruent. Two pathogenicity genes, *hil*A and *yscD*, were studied. The *hilA* gene was amplified in seven of the nine isolates, whereas the *ysc*D gene was amplified only in the positive control (type-strain). The 16S rDNA analysis using eight restriction enzymes allowed the grouping of strains with greater similarity, demonstrating a relevant genetic diversity. As expected, variability was highest in RAPD analysis, medium in sequencing of pathogenicity genes, and lowest in 16S rDNA restriction analysis.

Conflicts of interest

The authors declare no conflict of interest.

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