

Genetic characterization of *Chromobacterium* isolates from black water environments in the Brazilian Amazon

M. Hungria¹, S. Astolfi-Filho², L.M.O. Chueire¹, M.F. Nicolás³, E.B.P. Santos², M.R. Bulbol², A. Souza-Filho², E. Nogueira Assunção², M.G. Germano^{1,4} and A.T.R. Vasconcelos³

¹Embrapa Soja, Londrina, PR, ²Laboratório de Tecnologias de DNA, Centro de Apoio Multidisciplinar, Universidade Federal do Amazonas, Manaus, AM, ³Laboratório Nacional de Computação Científica, Rua Getúlio Vargas, Petrópolis, RJ, and ⁴M.Sc. at the Universidade Estadual de Londrina, PR, Brazil

2004/0435: received 18 April 2004, revised and accepted 4 March 2005

ABSTRACT

M. HUNGRIA, S. ASTOLFI-FILHO, L.M.O. CHUEIRE, M.F. NICOLÁS, E.B.P. SANTOS, M.R. BULBOL, A. SOUZA-FILHO, E. NOGUEIRA ASSUNÇÃO, M.G. GERMANO AND A.T.R. VASCONCELOS. 2005.

Aims: To isolate and to characterize the diversity of *Chromobacterium violaceum* from the Brazilian Amazon region.

Methods and Results: Twenty-two isolates were obtained from the waters and banks of the river Negro, in the Brazilian Amazon. All isolates were able to grow *in vitro* at 44°C and pH 4.0, but were adversely affected by temperatures below 15°C, and unable to survive at 4°C, properties that may be related to the adaptation to the ecosystem. The isolates were joined at a final level of similarity of only 13% in the rep-PCR analysis. The analysis of 16S rRNA genes resulted in three main groups clustered at a final level of similarity of 97% and only three isolates were clustered with the type strain. Similar data were obtained for the 23S rRNA gene.

Conclusions: A high level of genetic diversity was verified with indications that the Brazilian isolates would fit into at least two new clusters besides *C. violaceum* species.

Significance and Impact of the Study: The results show remarkable bacterial adaptability and genetic diversity of *C. violaceum* in the Amazon region.

Keywords: Amazon region, biodiversity, *Chromobacterium violaceum*, rep-PCR, 16S rRNA, 23S rRNA.

INTRODUCTION

Chromobacterium violaceum is a Gram-negative bacterium first described in 1882 (Boisbaudran 1882) and today classified in the Betaproteobacteria class, order Neisseriales, family Neisseriaceae (Garrity and Holt 2001). It is frequently found in soil and water in tropical and temperate regions and its most notable characteristic is the production of the purple pigment known as violacein (Strong 1944; Sneath 1984). Many studies performed with *C. violaceum* are related to the regulation of phenotypic characteristics, including production of violacein and cyanide by a quorum-sensing endogenous system in which the specific

autoinducer was identified as *N*-acyl homoserine lactone (AHL) (McClellan *et al.* 1997).

Chromobacterium violaceum is abundant in the black waters and on the banks of the river Negro (Black River basin, with 690 000 km²) in the Brazilian Amazon region and its therapeutic properties have been the object of study since the late 1970s (Caldas *et al.* 1978; Durán *et al.* 1989; Caldas 1990; Hungria *et al.* 2004). Violacein exhibits antimicrobial activity against *Mycobacterium tuberculosis* (de Souza *et al.* 1999), *Trypanosoma cruzi* (Caldas *et al.* 1978; Durán *et al.* 1989, 1994) and *Leishmania* sp. (Leon *et al.* 2001), important causes of endemic diseases especially in the Amazon region. It is well documented that violacein has other bactericidal (Lichstein and van de Sand 1945; Caldas 1990), anti-viral (Rettori and Durán 1998) and anti-tumoral (Ueda *et al.* 1994; Melo *et al.* 2000) activities. The biotechnological

Correspondence to: Mariangela Hungria, Embrapa Soja, Cx. Postal 231, CEP 86001-970, Londrina, Paraná, Brazil (e-mail: hungria@cnpsa.embrapa.br).

potential of *C. violaceum* includes also the capacity to synthesize bioplastics as the 3-hydroxyvalerate homopolymer (polyhydroxyvalerate) and other short-chain polyhydroxyalkanoates (PHAs) (Forsyth *et al.* 1958; Steinbüchel *et al.* 1993), as well as to hydrolyse plastic films (Gourson *et al.* 1999) and to solubilize gold (Smith and Hunt 1985; Campbell *et al.* 2001), among others.

The genome of *C. violaceum* strain ATCC 12472, isolated from fresh water in Mentekab, Malaysia, was completed by the Brazilian National Genome Project Consortium and revealed remarkable and exploitable bacterial adaptability (Vasconcelos *et al.* 2003). Strain ATCC 12472 was chosen by the consortium because it is the type strain (Sneath 1984) and has been used in several studies in Brazil over the past three decades (Caldas *et al.* 1978; Durán *et al.* 1989, 1994; de Souza *et al.* 1999; Melo *et al.* 2000; Leon *et al.* 2001). Although it represents an important component of the Amazonian ecosystem, no studies of the diversity of indigenous isolates have been reported. The work described here represents a first step in the characterization of the diversity of *C. violaceum* in Brazil.

MATERIALS AND METHODS

Isolates from black waters environments and reference strain

Water samples of river Negro were collected from four sites in/around Manaus city (latitude: $-03^{\circ}06'07''$, longitude: $-60^{\circ}01'30''$), the capital of the State of Amazonas: INPA's Campus, Tarumã Grande, Rio Preto da Eva and Presidente Figueiredo. Samples were taken in the raining season, from December to May, when water speed is around 2.5 km h^{-1} , with the pH ranging from 3.9 to 4.1 and the temperature from 25 to 26°C , while the temperature of the air may reach 40°C . Samples were poured into plates containing Luria-Bertani (LB) medium (Sambrook *et al.* 1989). Twenty-two isolates showing typical violet colonies in solid culture medium were selected. *Chromobacterium violaceum* type strain ATCC 12472, used as reference in all analyses, was obtained from the Fundação Tropical de Pesquisas André Tosello, Campinas, São Paulo, Brazil, where it is designated as CCT 3496. Other designations for the strain are NCIB 9131, NCTC 9757, JCM 1249, DSM 30191, IAM 12470, D 252 and LMG 1267.

Morphological and physiological characterization

Morphology of the colonies was evaluated in LB solid medium. Ability to grow at 4, 10, 15, 30, 37, 40 and 44°C , as well as to grow in buffered liquid medium with pH 4.0, 5.0 and 7.0 was verified as described before (Hungria *et al.* 2001).

DNA extraction

Total genomic DNA of each isolate was extracted from bacterial batch cultures grown in LB broth until late exponential phase ($10^9 \text{ cells ml}^{-1}$). To obtain clean DNA samples, the extraction procedure included the addition, for each 400 ml of bacterium resuspended in TE 50/20, of $50 \mu\text{l}$ of 10% SDS, $5 \mu\text{l}$ of proteinase K (20 mg ml^{-1}), $10 \mu\text{l}$ of lysozyme (5 mg ml^{-1}) and $2 \mu\text{l}$ of RNase (10 mg ml^{-1}).

Amplification by rep-PCR (BOX A1R)

A 50 ng of each DNA was used for the amplification by PCR with BOX A1R (5'-CTACGGCAAGGCGACGCTGACG-3') primer (Versalovic *et al.* 1994). Analyses were performed in an MJ Research Inc. PTC-100 thermocycler, and the cycles were as follows: an initial cycle of denaturation at 95°C for 7 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 65°C for 8 min; a final extension cycle of 65°C for 16 min; and a final soak at 4°C . The PCR products were visualized after electrophoresis on a $17 \times 20 \text{ cm}$ 1.5% agarose gel (low EED, type I-A) and photographed under UV light.

Clustering analysis of BOX A1R-PCR products

Clustering analysis of the BOX A1R-PCR products was performed using the Bionumerics program version 1.50 (Applied Mathematics, Kortrijk, Belgium), with the UP-GMA (unweighted pair-group method, with arithmetic mean) algorithm (Sneath and Sokal 1973) and the coefficient of Jaccard.

16S ribosomal DNA sequence analysis

The DNA samples were submitted to amplification of the whole 16S rDNA region, using the primers Y1, Y3 and intermediate primers, as described before (Chen *et al.* 2000). Direct sequencing of the PCR products was also performed as described before (Chen *et al.* 2000), in a Perkin-Elmer ABI 377 sequence analyser.

23S ribosomal DNA sequence analysis

The DNA samples of nine isolates were amplified with the primers P3 (5'-CCGTGAGGGAAAGGTGAAAAGTAC-3') and P4 (5'-CCCGCTTAGATGCTTTCAGC-3') as described by Terefework *et al.* (1998). Direct sequencing of the PCR products was performed as described for the 16S rDNA region to obtain partial sequences of the 23S rDNA region.

GenBank accession numbers and phylogenetic analysis

The 16S and 23S rDNA sequences generated were submitted to the GenBank database (<http://www.ncbi.nlm.nih.gov/blast>) to search for significant alignments. For the 16S rRNA sequences, the isolates from this study received the following accession numbers: 06 (AY117560.1), 07 (AY117557.1), 21 (AY117561.1), 23 (AY117556.1), 27 (AY117562.1), 29 (AY117558.1), 32 (AY117563.1), 44 (AY117564.1), 46 (AY117559.1), 48 (AY117552.1), 49 (AY117553.1), 52 (AY117554.1), 53 (AY117565.1), 62 (AY117566.1), 64 (AY117559.1), 66 (AY117567.1), 67 (AY117568.1), 68 (AY117569.1), 69 (AY117570.1), 70 (AY117571.1), 71 (AY117572.1) and 72 (AY117574.1). The sequences were aligned pairwise with those of the following strains (GenBank accession numbers in parentheses): ATCC 12472 (AE016921.1), MBIC 3901 (AB017487) and DSM 30191 (AJ247211). For the 23S rRNA sequences, the isolates 23, 29, 32, 44, 48, 52, 53, 64 and 71 from this study received the accession numbers AY466058–AY466066, respectively, and the sequences were aligned pairwise with that of the strain ATCC 12472 (AE016921.1). Phylogeny trees were inferred with the UPGMA algorithm using the Bionumerics program. One thousand bootstrap resamplings were used to evaluate robustness of the inferred trees (Felsenstein 1985).

Detection of genes of pathogenicity

The DNAs of the isolates were also amplified with five of the 14 primers related to pathogenicity previously used for *C. violaceum* strain ATCC 12472 and other isolates from the State of Minas Gerais, Brazil (Vasconcelos *et al.* 2003). Primers used were related to the type III secretion system: *fliC* (5'-CAAGACCCATAACGCCAC-3'; 5'-ATGGTGT TGAGGTCGCTT-3'; annealing temperature of 60°C, 30 cycles; expected product of 596 bp); *hylD* (5'-CTTC CTACCCGCCACC-3'; 5'-TTTCCTCGCTCAGTTG CTT-3'; annealing temperature of 60°C, 30 cycles; expected product of 430 bp); *invB* (5'-GACAACCATTCACCAT C-3'; 5'-TCCAGGCTGTCGAAGAAG-3'; annealing temperature of 60°C, 30 cycles; expected product of 430 bp); *spaQ* (5'-CCTGGTGCTGATGATGTC-3'; 5'-CAAGCC GCATCACTTCG-3'; annealing temperature of 60°C, 30 cycles; expected product of 205 bp) and *sseD* (5'-TACCA ACGTCCCACAGC-3'; 5'-ACAGCCCCGTGGACAT C-3'; annealing temperature of 60°C, 30 cycles; expected product of 329 bp).

RESULTS AND DISCUSSION

Twenty-two isolates were obtained from the black waters of river Negro, at four sites (Table 1) and some morphological

Table 1 Origin of the isolates of *Chromobacterium violaceum*, colour of the colonies in LB solid medium and growth in liquid media with different pH and at different temperatures

Isolate/strain	Origin	Colour	Grow		
			15°C*	44°C*	pH 4.0†
06	Tarumã Grande	Light violet	-	+	+
07	Tarumã Grande	Violet	+	++	+
21	Presidente Figueiredo	Violet	-	+	+
23	Presidente Figueiredo	Violet	+	+	+
27	Presidente Figueiredo	Violet	+	+	++
29	Presidente Figueiredo	Violet	-	+	+
32	Presidente Figueiredo	Violet	-	++	++
44	INPA's Campus	Violet	-	+	+
46	INPA's Campus	Violet	+	+	+
48	Rio Preto da Eva	Violet	-	+	+
49	Rio Preto da Eva	Violet	+	+	+
52	Rio Preto da Eva	Violet	-	+	+
53	Rio Preto da Eva	Light violet	+	+	+
62	Rio Preto da Eva	Light violet	-	+	+
64	Rio Preto da Eva	Light violet	+	+	+
66	Rio Preto da Eva	Violet	-	+	+
67	Rio Preto da Eva	Violet	-	+	+
68	Rio Preto da Eva	Violet	+	+	+
69	Rio Preto da Eva	Violet	-	+	+
70	Rio Preto da Eva	Violet	-	+	+
71	Rio Preto da Eva	Violet	+	+	+
72	Rio Preto da Eva	Violet	-	+	+
ATCC12472 ^T	Mentakab, Malaysia	Violet	+	-	-

*None of the indigenous isolates was able to grow at 4 or 10°C and all grew at 30, 37 and 40°C.

†All indigenous isolates grew at pH 5.0 and 7.0.

and physiological properties were evaluated. All isolates showed properties typical of *C. violaceum* such as colony morphology, production of cyanide *in vitro* and violet pigmentation on solid medium (Sneath 1984). However, the violet colour of four isolates (06, 53, 62 and 64) was lighter than that of the others (Table 1). Maximum temperature for growth of the species is usually described as 40°C, although 20% of strains were previously found to grow at 44°C (Sneath 1984). All indigenous isolates were able to grow at 44°C, a feature probably related to the high temperatures in the Amazon region. Furthermore, the minimum temperature for growth of the species was previously described as from 10 to 15°C (Sneath 1984), although isolation of *C. violaceum* from colder regions has been reported (Kriss *et al.* 1976). The Amazon isolates were extremely sensitive to temperatures below 15°C and unable to survive at 4°C (Table 1). Finally, Sneath (1984) reported absence of growth at pH below 5,

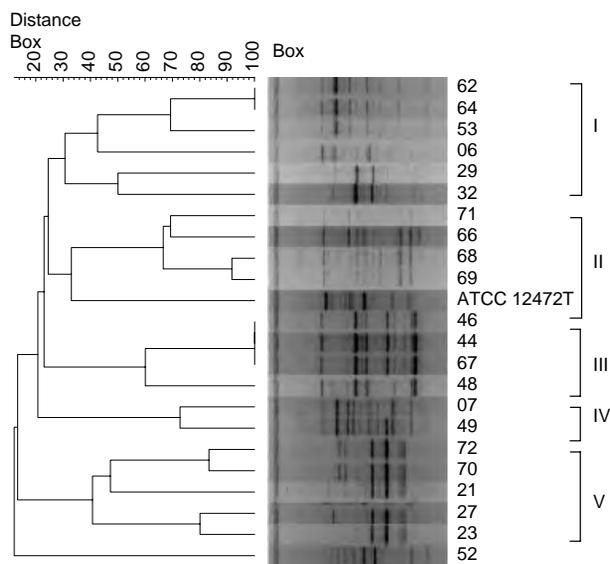


Fig. 1 Dendrogram of *Chromobacterium* isolates from river Negro and of *Chromobacterium violaceum* ATCC 12472^T based on clustering of BOX A1R-PCR products using the UPGMA algorithm and the Jaccard coefficient

whereas the Amazon isolates were able to grow at pH 4.0 (Table 1), which may also reflect environmental adaptability, as the pH of the river Negro ranges from 3.8 to 4.9 (Walker 1990; Hungria *et al.* 2004).

Interspersed repetitive sequences, usually localized in the intergenic space of prokaryotic genomes, as the BOXAIR sequences are often highly conserved among dissimilar bacteria and can be used to evaluate diversity (de Bruijn 1992; Versalovic *et al.* 1994). In this study, when the DNAs of the Brazilian isolates and of the type strain were analysed by the BOX A1R-PCR, five main groups were defined and the isolates were clustered at a very low level of similarity (Fig. 1). In the upper part of the dendrogram, group I included isolates 62, 64 and 53, from Rio Preto da Eva, showing several bands in common and joined at a level of similarity of 69%. Isolate 06, from Tarumã Grande, was clustered into this group with a similarity of 43%. Those four isolates showed a lighter violet colour in LB solid medium (Table 1). Finally, two other isolates, 29 and 32, from Presidente Figueiredo, were 50% similar and were linked to the other strains at a level of 31% (Fig. 1). Group II included four isolates, 71, 66 and two others highly similar, 68 and 69 (92%), all from Rio Preto da Eva and clustered at a 66% level of similarity; in view of the high similarity, both pairs of isolates may represent variants of the same strain. Type strain ATCC 12472 joined this group with a similarity of 33%. Three isolates, 46, 44 and 67, showed identical repetitive sequences and may represent the same strain. Furthermore, the first two isolates came from INPA's Campus and the third from Rio Preto da Eva,

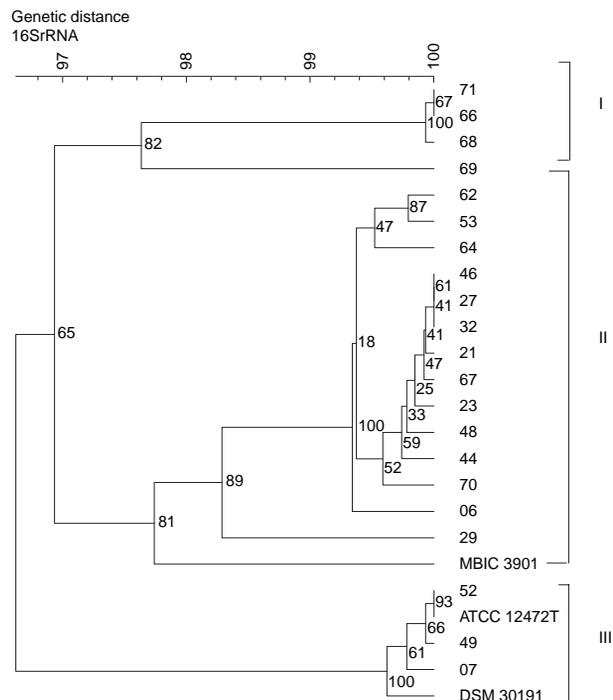


Fig. 2 Dendrogram built by the use of the UPGMA algorithm with the aligned whole 16S rRNA sequences of *Chromobacterium* isolates from river Negro and of strains used as references, including *C. violaceum* ATCC 12472^T. GenBank accession numbers are given in the text. One thousand bootstrap resamplings were used to evaluate robustness of the inferred trees

indicating that the strain was not restricted to one site. Isolate 48, also from Rio Preto da Eva, clustered with those isolates with a similarity of 60%, completing group III. Group IV consisted of two isolates, 07 and 49, from Tarumã Grande and Rio Preto da Eva, respectively, showing 73% of similarity. The last group (V) consisted of two subgroups, the first one with isolates 72 and 70, both from Rio Preto da Eva, highly similar (83%) and linked to isolate 21, from Presidente Figueiredo, with a similarity of 47%. The other subgroup included isolates 21 and 27, both from Presidente Figueiredo, which also showed high similarity (80%). Finally, the most disparate isolate, 52, from Rio Preto da Eva, joined the other groups at a similarity of only 13%. In conclusion, the rep-PCR analysis indicated high genetic diversity among the Amazonian isolates, which were quite distinct from the type strain. In general, the clusters included strains from more than one site and some isolates showing high similarity were obtained from different sites, indicating broad distribution of strains across the ecosystem.

The results achieved in the analysis of the whole 16S rRNA gene (isolate 72 was not included) confirmed the genetic diversity of the isolates (Fig. 2). Three main groups were observed. The first one clustered four isolates from Rio

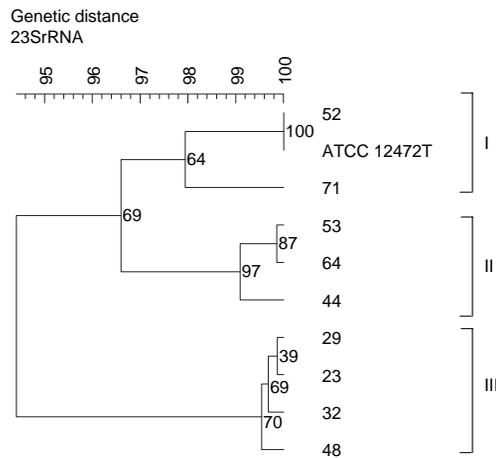


Fig. 3 Dendrogram built by the use of the UPGMA algorithm with the aligned partial 23S rRNA sequences of *Chromobacterium* isolates from river Negro and of strains used as references, including *C. violaceum* ATCC 12472^T. GenBank accession numbers are given in the text. One thousand bootstrap resamplings were used to evaluate robustness of the inferred trees

Preto da Eva with a similarity of 99.9%. Those isolates were also clustered in the same group in the rep-PCR analysis (group II) (Fig. 1). The majority of the Brazilian isolates fit into group II of the 16S rRNA analysis; 14 were clustered with a 98.2% level of similarity (Fig. 2). A first subgroup included three isolates that were joined at a similarity of 99.6%; they were also clustered in group I with rep-PCR analysis (Fig. 1), and produced a light violet colour in culture (Table 1). Although they showed high similarity for the ribosomal region, the other isolates within 16S rRNA group II occupied different positions in the rep-PCR analysis, including groups I, III and V (Fig. 1). It is also noteworthy that strains with identical rep-PCR profiles, 62 and 64 and also 46, 44 and 67, were not identical in the 16S ribosomal sequence analysis. The most similar known strain to this group was MBIC 3901. The last group of the 16S rRNA analysis included the type strain as well as the strain DSM 30191 (accession number AB017487) isolated at the Japan Marine Biotechnology Institute (Fig. 2). Three Brazilian isolates from two sites, Rio Preto da Eva and Tarumã, fit into group III and two of them, 07 and 49, were also quite similar in the rep-PCR analysis; on the contrary, isolate 52, with complete identity of bases with ATCC 12472, showed higher dissimilarity in relation to all other isolates in the rep-PCR analysis. The isolates and strains within this last group were joined with a level of similarity of 99.7%. The three groups of 16S rRNA region were clustered at a level of similarity of 97.7%, therefore the results indicate that the Brazilian isolates fit into two new clusters besides the one including the species *C. violaceum*.

The comparison between the phylogenies obtained with rep-PCR products with the 16S rRNA sequences shows only a partial agreement in the grouping of isolates. The group showing light violet colour in culture medium was clustered in both analyses. Group III of the 16S rRNA included isolate 52 and the type strain, quite dissimilar to all other isolates in the rep-PCR analysis and two isolates (49 and 07) that showed no correlation with the rep-PCR clustering. As pointed before, the pattern of conservation of a repeated element may not correlate with established phylogeny (Versalovic *et al.* 1994), thus rep-PCR indicates the diversity of the isolates, while the 16S rRNA indicates their phylogenetic relationship.

Analysis of the 23S rRNA ribosomal gene was shown to be useful for taxonomic purposes (Olsen and Woese 1993; Ludwig and Schleifer 1994). In this study, partial sequences of the 23S ribosomal region were obtained for nine Brazilian isolates. The phylogenetic tree obtained confirmed the complete identity of bases of the Brazilian isolate 52 and the type strain ATCC 12472 and isolate 71, previously in group I of the 16S rRNA analysis clustered in this first group with a final level of similarity of 98% (Fig. 3). The other isolates analysed, all positioned in group II in the 16S rRNA analysis (Fig. 2), were split into two groups when the 23S rRNA gene was considered (Fig. 3). Group II included three isolates from two different sites, clustered with a similarity of bases of 99.1%, and the group was joined to group I with a similarity of 96.6% (Fig. 3). The similarity of the bases within the third group was high (99.5%), but it was linked to the other groups with a lower similarity (94.4%). Differences in clustering when considering the 16S or the 23S rRNA region have been reported before (van Berkum *et al.* 2003) and in this study, the higher diversity of bases verified for the 23S rRNA region resulted a new cluster. Furthermore, when compared with the type strain, the lower level of similarity of bases of the 23S rRNA region confirms that the Brazilian isolates would fit into another species besides the already described *C. violaceum*. The 23S rRNA clustering (Fig. 3) also did not correlate with the results obtained in the rep-PCR analysis (Fig. 1).

When the 16S rRNA and 23S rRNA genes were analysed together (data not given), the dendrogram obtained was quite similar to that for the 23S rRNA region alone. Isolate 52 was identical to type strain ATCC12472 and both were clustered with isolate 71 (98% of similarity). Group II included isolates 53, 64 and 44 (99.1% similarity), and was linked to group I at a 97% level of similarity. Finally, isolates 32, 23, 48 and 29 were clustered with a similarity of 99.5% and joined the other groups with a similarity of bases of 97%.

Although *C. violaceum* is considered to be a saprophyte, it may also be a pathogen of animals and man (Richard 1993). Human infections generally occur in immunodepressed individuals, however, as discussed by Vasconcelos *et al.* (2003), the local population drink the river Negro water without being infected. The complete genome of strain

Table 2 Amplification of five genes related to pathogenesis in the Amazon isolates and in the type strain of *Chromobacterium violaceum*

Isolate/strain	<i>fliC</i>	<i>hylD</i>	<i>invB</i>	<i>spqA</i>	<i>sseD</i>
06	-	-	-	-	-
07	+	-	-	+	+
21	+	-	-	-	-
23	+	-	-	-	+
27	-	-	-	-	-
29	-	-	-	-	-
32	+	-	-	-	+
44	-	-	-	-	+
46	-	-	-	-	+
48	+	-	-	-	-
49	+	-	-	+	+
52	+	-	-	+	+
53	-	-	-	-	+
62	-	-	-	-	+
64	-	-	-	-	-
66	+	-	-	+	+
67	+	-	-	+	+
68	+	-	-	+	-
69	-	-	-	-	-
70	-	-	-	+	+
71	-	-	-	-	-
72	-	-	-	+	+
ATCC12472 ^T	+	+	+	+	+

ATCC 12472 was found to contain several ORFs encoding type-III secretory systems in pathogenic islands similar to those in *Salmonella typhimurium* and *Yersinia pestis*, but some key genes were missing (Vasconcelos *et al.* 2003). In PCR-based assays with isolates from the State of Minas Gerais, Brazil, 14 genes related to pathogenesis were detected in some isolates but not in others. In this study, five of those primers were assayed with the Amazonian isolates but the amplification occurred in only a few (Table 2). In particular, *invB*, related to a protein responsible for the invasion of host cells (Collazo *et al.* 1995) and the regulator *HylD*, related to the expression of invasion genes (Bajaj *et al.* 1995) were detected in the type strain but were absent from all isolates, including the highly similar isolate 52. That observation is consistent with the fact that *Chromobacterium* is widely spread in the Amazon region but not a public-health concern.

In summary, isolates from the waters of river Negro in Brazil that had characteristics of *C. violaceum* were found to have a high level of genetic diversity. Analyses of the 16S and 23S ribosomal genes indicated that they would fit into at least two new clusters besides that including the described *C. violaceum*. Furthermore, all of the river Negro isolates were tolerant to high temperature (44°C) and acid conditions (pH 4.0) *in vitro*, reflecting their adaptability to the Amazonian ecosystem. In most Amazonian strains, pathogenic genes identified in the genome of the type strain

ATCC 12472 were not detected. We are now investigating other genes, aiming to better understand the diversity of *Chromobacterium* in Brazil.

ACKNOWLEDGEMENTS

To Ministério da Ciência e Tecnologia (MCT)/Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support (680220/00-3).

To Fábio Luís Mostasso, for helping in the cluster analyses and Dr Allan R.J. Eaglesham, for suggestions on the manuscript. The authors received fellowships from CNPq.

REFERENCES

- Bajaj, V., Hwang, C. and Lee, C.A. (1995) *hilA* is a novel *ompR/toxR* family member that activates the expression of *Salmonella typhimurium* invasion genes. *Mol Microbiol* **18**, 715–727.
- van Berkum, P., Terefework, Z., Paulin, L., Suomalainen, S., Lindström, K. and Eardly, B.D. (2003) Discordant phylogenies within the *rrn* loci of rhizobia. *J Bacteriol* **185**, 2988–2998.
- Boisbaudran, L. (1882) Matière colorante se formant dans la colle de farine. *C R Biol* **94**, 562–563.
- de Bruijn, F.J. (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl Environ Microbiol* **58**, 2180–2187.
- Caldas, L.R. (1990) Um pigmento nas águas negras. *Ci Hoje* **11**, 55–57.
- Caldas, L.R., Leitão, A.A.C., Santos, S.M. and Tyrrell, R.M. (1978) Preliminary experiments on the photobiological properties of violacein. In *Proceedings of the International Symposium on Current Topics in Radiology and Photobiology* ed. Tyrrell, R.M. pp. 121–126. Rio de Janeiro: Academia Brasileira de Ciências.
- Campbell, S.C., Olson, G.J., Clark, T.R. and McFeters, G. (2001) Biogenic production of cyanide and its application to gold recovery. *J Ind Microbiol Biotechnol* **26**, 134–139.
- Chen, L.S., Figueredo, A., Pedrosa, F.O. and Hungria, M. (2000) Genetic characterization of soybean rhizobia in Paraguay. *Appl Environ Microbiol* **66**, 5099–5103.
- Collazo, C.M., Zierler, M.K. and Galan, J.E. (1995) Functional analysis of the *Salmonella typhimurium* invasion genes *invI* and *invJ* and identification of a target of the protein secretion apparatus encoded in the *inv* locus. *Mol Microbiol* **15**, 25–38.
- Durán, N., Campos, V., Riveros, R., Joyas, A., Pereira, M.F. and Haun, M. (1989) Bacterial chemistry: III. Preliminary studies on the trypanosomal activities of *Chromobacterium violaceum* products. *An Acad Bras Cienc* **61**, 31–36.
- Durán, N., Antonio, R.V., Haun, M. and Pilli, R.A. (1994) Biosynthesis of a trypanocide by *Chromobacterium violaceum*. *World J Microbiol Biotechnol* **10**, 686–690.
- Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Forsyth, W.G.C., Hayward, A.C. and Roberts, J.B. (1958) Occurrence of poly- β -hydroxybutyric acid in aerobic gram-negative bacteria. *Nature* **182**, 800–801.

- Garrity, G.M. and Holt, J.G. (2001) The road map to the Manual. In *Bergey's Manual of Systematic Bacteriology* ed. Boone, D.R., Castenholz, R.W. and Garrity, G.M. pp. 119–166. New York: Springer.
- Gourson, C., Benhaddou, R., Granet, R., Krausz, P., Verneuil, B., Branland, P., Chauvelon, G., Tribault, J.F. *et al.* (1999) Valorization of maize bran to obtain biodegradable plastic films. *J Appl Polym Sci* **74**, 3040–3045.
- Hungria, M., Chueire, L.M.O., Coca, R.G. and Megías, M. (2001) Preliminary characterization of fast growing rhizobial strains isolated from soybean nodules in Brazil. *Soil Biol Biochem* **33**, 1349–1361.
- Hungria, M., Nicolás, M.F., Guimarães, C.T. and Vasconcelos, A.T.R. (2004) Tolerance to stresses and environmental adaptability of *Chromobacterium violaceum*. *Gen Mol Res* **3**, 102–116.
- Kriss, A.E., Mitskevich, I.N., Rozanova, E.P. and Osnitskaia, L.K. (1976) [Microbiological studies of the Wanda Lake (Antarctica)]. *Mikrobiologiya* **45**, 1075–1081.
- Leon, L.L., Miranda, C.C., de Souza, A.O. and Durán, N. (2001) Antileishmanial activity of the violacein extracted from *Chromobacterium violaceum*. *J Antimicrob Chemother* **48**, 449–450.
- Lichstein, H.C. and van de Sand, V.F. (1945) Violacein, an antibiotic pigment produced by *Chromobacterium violaceum*. *J Infect Dis* **76**, 47–51.
- Ludwig, W. and Schleifer, K.H. (1994) Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol Rev* **15**, 155–173.
- McClellan, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H. *et al.* (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **143**, 3703–3711.
- Melo, P.S., Maria, S.S., Vidal, B.C., Haun, M. and Durán, N. (2000) Violacein cytotoxicity and induction of apoptosis in V79 cells. In *Vitro Cell Dev Biol Anim* **36**, 539–543.
- Olsen, G.J. and Woese, C.R. (1993) Ribosomal RNA: a key to phylogeny. *FASEB J* **7**, 113–123.
- Rettori, D. and Durán, N. (1998) Production, extraction and purification of violacein: an antibiotic pigment produced by *Chromobacterium violaceum*. *World J Microbiol Biotechnol* **14**, 665–668.
- Richard, C. (1993) *Chromobacterium violaceum*, opportunist pathogenic bacteria in tropical and subtropical regions. *Bull Soc Pathol Exot* **86**, 169–173.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Smith, A.D. and Hunt, R.J. (1985) Solubilization of gold by *Chromobacterium violaceum*. *J Chem Technol Biotechnol, Part B, Biotechnology* **35**, 110–116.
- Sneath, P.H.A. (1984) Genus *Chromobacterium* Bergonzini 1881, 153^{AL}. In *Bergey's Manual of Systematic Bacteriology* ed. Krieg, N.H. (vol. 1) and Holt, J.G. (ed.-in-chief). pp. 580–582. Baltimore: Williams & Wilkins.
- Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy: The Principles and Practice of Numerical Classification*. San Francisco: W.H. Freeman & Co.
- de Souza, A.O., Aily, D.C.G., Sato, D.N. and Durán, N. (1999) Atividade da violaceína *in vitro* sobre o *Mycobacterium tuberculosis* H37RA. *Rev Inst Adolfo Lutz* **58**, 59–62.
- Steinbüchel, A., Debzi, E.-M., Marchessault, R.H. and Timm, A. (1993) Synthesis and production of poly(3-hydroxyvaleric acid) homopolymer by *Chromobacterium violaceum*. *Appl Microbiol Biotechnol* **39**, 443–449.
- Strong, F.M. (1944) Isolation of violacein. *Science* **100**, 287.
- Terefework, Z., Nick, G., Suomalainen, S., Paulin, L. and Lindström, K. (1998) Phylogeny of *Rhizobium galegae* with respect to other rhizobia and agrobacteria. *Int J Syst Bacteriol* **48**, 349–356.
- Ueda, H., Nakajima, H., Hori, Y., Goto, T. and Okuhara, M. (1994) FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* no. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties. *J Antibiot* **47**, 301–310.
- Vasconcelos, A.T.R., Almeida, D.F., Hungria, M., Guimarães, C.T., Antônio, R.V., Almeida, F.C., Almeida, L.G.P., Almeida, R. *et al.* (2003) The complete genome sequence of *Chromobacterium violaceum* reveals remarkable and exploitable bacterial adaptability. *Proc Natl Acad Sci USA* **100**, 11660–11665.
- Versalovic, J., Scheider, M., de Bruijn, F.J. and Lupski, J.R. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol and Cell Biol* **5**, 25–40.
- Walker, I. (1990) Ecologia e biologia dos igapós e iguarapés. *Ci Hoje* **11**, 44–53.