Research Article

Diversity and three-dimensional structures of the alpha Mcr of the methanogenic Archaea from the anoxic region of Tucuruí Lake, in Eastern Brazilian Amazonia

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Abstract

Methanogenic archaeans are organisms of considerable ecological and biotechnological interest that produce methane through a restricted metabolic pathway, which culminates in the reaction catalyzed by the Methyl-coenzyme M reductase (Mcr) enzyme, and results in the release of methane. Using a metagenomic approach, the gene of the α subunit of *mcr* (*mcr*α) was isolated from sediment sample from an anoxic zone, rich in decomposing organic material, obtained from the Tucuruí hydroelectric dam reservoir in eastern Brazilian Amazonia. The partial nucleotide sequences obtained were 83 to 95% similar to those available in databases, indicating a low diversity of archaeans in the reservoir. Two orders were identified - the *Methanomicrobiales*, and a unique Operational Taxonomic Unit (OTU) forming a clade with the *Methanosarcinales* according to low bootstrap values. Homology modeling was used to determine the three-dimensional (3D) structures, for this the partial nucleotide sequence of the mcrα were isolated and translated on their partial amino acid sequences. The 3D structures of the archaean Mcrα observed in the present study varied little, and presented approximately 70% identity in comparison with the Mcrα of *Methanopyrus klanderi*. The results demonstrated that the community of methanogenic archaeans of the anoxic C1 region of the Tucurui reservoir is relatively homogeneous.

Key words: Amazonian, Archaea, diversity, methyl-coenzyme M reductase, mcrα, homology modeling.

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Introduction

The organisms of the Domain Archaea (Woese *et al.*, 1990) constitute a considerable proportion of the prokaryotes found in both aquatic and terrestrial ecosystems, where they are active in the carbon biogeochemical cycle. From a physiological viewpoint, the Domain is divided into three different groups: the haloarchaea, methanogenics, and thermophilics, the latter being dependent on sulfur (Valentine, 2007).

The methanogenic archaea are of special interest because of their capacity to reduce CO_2 and other compounds in the presence of H_2 (Conrad, 1999), under anoxic conditions, to produce methane through a process known as methanogenesis (Zehnder and Brock, 1979). Methane is one of the greenhouse gases and, with an atmospheric half-life of only 8.9 years, it inflicts an effect on global warming 24 times greater than that contributed by CO_2 (Houghton *et al.*,

2001). As a non-fossil hydrocarbon, it also generates interest as a potential biofuel (Hansen *et al.*, 2006; Blottnitz and Curran 2007; Tilche and Galatola, 2008).

Archaean communities are distributed worldwide, and are known for their considerable heterogeneity (Schleper *et al.*, 2005). Studies of the diversity of archaean communities have been conducted in a variety of environments, from hot (Martinez *et al.*, 2006) and hypersaline (Oren, 2002) lakes, anoxic zones (Lehours *et al.*, 2007) and marine sediments (Teske and Sørensen, 2008) to the digestive systems of ruminants and humans (Lange and Ahring, 2001).

The reservoirs of hydroelectric power stations are known to emit large quantities of methane from biological sources, due to their tendency to accumulate organic material and create an environment favorable for methanogenesis (St Louis *et al.*, 2000; Kemenes *et al.*, 2007). With the World's most extensive system of river basins at its disposal, the Brazilian electricity network is centered on hydropower, which was responsible for 77.4% (374.015 GWh) of the electrical energy produced in the

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country in 2007 (Brazilian Ministry for Mines and Energy, 2009).

The country's main hydropower potential is in its northern region, along the tributaries of the Amazon basin. The construction of hydroelectric dams in the Brazilian Amazon over the past three decades has been the subject of considerable controversy between government agencies and many sectors of Society. By 2010, the Brazilian Ministry for Mines and Energy plans the construction of further three major dams, which will feature increased productivity, reduced flooding, and minimal impact on local fauna, flora, and indigenous societies, in comparison with previous projects.

Currently, the Tucuruí dam (UHE Tucuruí), which is located on the Tocantins River in the southeast of the Brazilian state of Pará (03°455'8" S, 49°40'21" W), is the country's largest and most productive hydropower plant. The Tucuruí Reservoir is 170 km long, with a perimeter of 7700 km, a depth of 60 m, and a total of 1500 islands (Tundisi, 2007).

Analyses conducted by Centrais Elétricas do Norte do Brazil, the state company responsible for Tucuruí hydroelectric power plant, as part of its quality control program, has indicated the existence of specific areas, such as Region C1 (Figure 1), which are favorable for methanogenesis. During most of the year, C1 has an anoxic environment, with still water and abundant organic material. Culture-independent analyses based on ssuRNA sequences have confirmed the presence of methanogenic archaea at various levels in the water column (Graças *et al.*, personal communication), and previous studies of methane emissions (Fearnside, 1997; Lima, 2005) have confirmed the elevated methanogenic capacity of the reservoir.

Figure 1 - Map showing the reservoir of the Tucuruí Hydroelectric Reservoir on the lower Tocantins in the Brazilian state of Pará and the study area at C1, where the specimens were collected.

Studies of the characteristics and diversity of archaean communities have been based on the nucleotide diversity of 16S rRNA (Keough *et al.*, 2003; Lehours *et al.*, 2005; Schleper *et al.*, 2005). Studies (Shapiro *et al.*, 2007) have also demonstrated the importance of the secondary structure of 16S for the analysis of the diversity and phylogenetic relations of prokaryotes. Similarly, analysis of the nucleotide sequence of the Methyl-coenzyme M reductase (*mcr*) gene has proven to be an invaluable tool for the study of diversity, given that this gene is present in all methanogenic archaea (Bapteste *et al.*, 2005).

Homology modeling is a well-known analytical tool through which it is possible to modeled the three-dimensional (3D) structure of a given protein based on knowledge of its primary structure (Martí-Renom *et al.*, 2000). This approach is only possible because the 3D structure of homologous proteins is conserved during the evolutionary process, especially in the case of the functional residuals, given that conservation of the structure is crucial to the maintenance and development of specific functions (Höltje *et al.*, 2003). Therefore, the strategy of this approach is based on the fact that, during the evolutionary process, the structural configuration of a protein tends to be better preserved than its aminoacid sequence, and that minor changes in the sequence generally result in only slight modifications of the 3D structure (Nayeem *et al.*, 2003). The comparative or homology modeling of the 3D structure of the target protein is possible if at least one resolved 3D structure sequence which is homologous to the amino acid sequence is available (Höltje *et al.*, 2003).

In the present study the diversity of archaean communities derived from samples of sediment collected from C1 region of UHE Tucuruí was accessed. In order to achieve, partial nucleotide sequences of *mcr*α were analysed and homology modeling was carried out.

Materials and Methods

Study site and sampling

Individual samples of sediment were collected during the dry season (July-November) of 2006 at point C1 of the Tucuruí Reservoir, using a Van Veen grab. *In loco* measurements of pH (6.25), temperature (30.2 °C) and conductivity (2.04 mg/mL) and local depth (20 m) were taken. The samples were stored at room temperature in flasks shielded from sunlight. The DNA was obtained using an UltraClean (Mo Bio, USA) kit, according to the manufacturer's instructions.

Amplification via PCR and cloning

Specific DNA sequences were amplified using PCR, with the degenerate primers ME1, 5'-GCMATGCARA THGGWATGTC-3', and ME2 5'-TCATKGCRTAGTT DGGRTAGT-3', as described by Hales *et al.* (1996), and MLf 5'- GGTGGTGTMGGATTCACACARTAYGCWA

CAGC -3', and MLr 5'- TTCATTGCRTAGTTWGGRT AGTT -3', as described by Luton *et al.* (2002). Each amplification solution contained 200 mM of Tris-HCl (pH 8.4), 500 mM of KCl, 200 μM of each dNTP, 3 μM of MgCl$_2$, 3.2 pmol of oligonucleotide, 100 ng of DNA and 1 U of Taq polymerase (Invitrogen, Brazil) in a final volume of 25 μL. The following cycling conditions were applied in an Eppendorf Mastercycler thermocycler (Eppendorf, Germany): initial denaturation at 95 °C for 6 min, 35 cycles at 95 °C for 1 min, annealing at 51 °C for 1 min, 72 °C for 1 min, followed by final extension at 72 °C for 5 min.

The amplicons were purified in agarose gel using an Illustra GFX PCR DNA kit and a Gel Band Purification Kit (GE Healthcare Life, UK). The fragments separated by electrophoresis in the 750 bp and 490 bp size range, corresponding to the amplicons generated using the ME and ML primers, respectively, were cloned in a pGEM®-T Easy Vector System (Promega Corp, USA) and electrotransformed into competent Top10 *Escherichia coli* cells (Invitrogen, USA). One hundred clones were selected randomly by screening of white colonies. The recombinant clones were transferred to Luria-Bertani culture medium (DIFCO, USA), and the plasmidial DNA was isolated by minipreparation and purified using MAGV N22 membranes (Millipore, USA).

Sequencing and the editing of the sequences

The purified DNA was sequenced in a MegaBACE 1000 automatic sequencer (GE HealthCare, USA). The sequences were then edited in BioEdit, version 7.05 (Hall 1999), aligned with ClustalW, version 2.0 (Thompson *et al.* 1994) and then checked using NCBI's BLAST tool.

Analyses of diversity

For the analysis of diversity, rarefaction curves were generated using version 1.3 of the Dotur program (Schloss and Handelsman, 2005) for the identification of unique Operational Taxonomic Units (OTUs) defined by a sequence dissimilarity of 3%. Two rarefaction curves were generated, one of which was used for homology modeling, the other including all the sequences obtained in this study. The first curve was produced using the amplicons obtained with the primers as described by Hales *et al.* (1996), whereas for the second, the amplicons obtained using the primers as described by Luton *et al.* (2002) were added.

The nucleotide sequences were converted into amino acid sequences using BioEdit software. Additionally, gene trees were produced in MEGA 4.1, using only the unique OTUs identified from either nucleotide or amino-acid sequences. For this, the Kimura 2-parameter evolutionary model was used with bootstrap analysis for 1000 replicates.

Homology modeling

All procedures of homology modeling follow four main stages (Schwede *et al.*, 2003): (i) selection of the protein template; (ii) alignment of the target sequence and template protein; (iii) construction of the 3D model; (iv) and evaluation of the 3D model.

For the homology modeling, the partial nucleotide sequences obtained with the ME primer were chosen as a target sequence, because they produced a fragment of 750 bp, which stretches from the amino acid 227-alanine to 476-proline, forming a total of 249 fundamental residuals for the enzymatic function of the Mcrα protein. The partial nucleotide sequences were converted into amino acid sequences and the multiple alignment was carried out using software ClustalW.

The 3D models were modeled by the rigid body grouping method, using the Swiss-Model Workspace (Schwede *et al.*, 2003; Arnold *et al.*, 2006). This server is used to align the target sequences and template structure available in the Protein Data Bank (PDB). Once the template has been selected the 3D structure of the target sequences can be modeled.

The stereochemical quality of the structures was evaluated using ProCheck, version 3.0 (Laskowski *et al.*, 2003), and the distribution of residual energy in Prosa, version 3.0 (Wiederstein and Sippl, 2007). Following this evaluation, the target structures were superimposed in the *UCSF* Chimera program, version 1.21 (Meng *et al.*, 2006), in order to identify more accurately any differences in folding that may exist among the structures within the different clades represented in the gene trees. The Root Mean Square (RMS) was also calculated to compare the target and template structures.

GeneBank® access: All sequences are available in GenBank® database as accession number FJ715506 - FJ715617. The sequences used to build the 3D structures are available as FJ715545, FJ715553 - FJ715558, FJ715569, FJ715571, FJ715573 - FJ715577, FJ715579, FJ715581, FJ715582, FJ715584 - FJ715587, FJ715594, FJ715595, FJ715597 - FJ715599, FJ715605 - FJ715607.

Results

Analyses of nucleotide diversity

The first rarefaction curve (Figure 2A) produced 18 OTUs from 28 archaean nucleotide sequences obtained using the primers according to Hales *et al.* (1996) following treatment with Dotur. For the second curve (Figure 2B), we added, to the original 28 sequences, the sequences obtained using the primers according to Luton *et al.* (2002) resulting in a total of 113 nucleotide sequences, by which Dotur identified 58 unique OTUs. The nucleotide (Figure 3A) and amino acid (Figure 3B) trees generated by the MEGA program from the 18 unique OTUs are both characterized by three distinct clades, although the nucleotide cladogram returned higher bootstrap values.
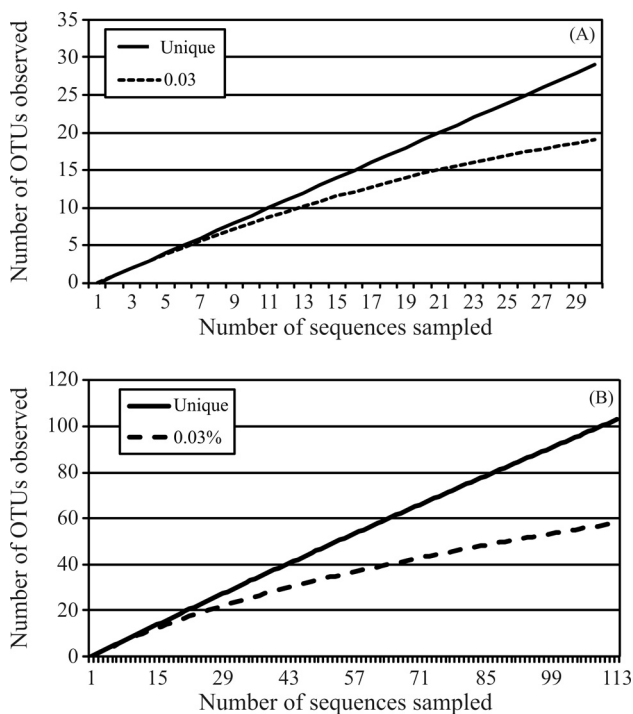
Figure 2 - Rarefaction curve for the *mcrα* gene (A) First rarefaction curve. The dotted line represents the sample from the present study, and the continuous line, the statistical estimate of the unique OTUs observed based on the differences in nucleotide sequences observed in the sample. (B) Second rarefaction curve plotted following the inclusion of the sequence data for the *mcrα* gene obtained using the primers of Hales *et al.* (1996) and Luton *et al.* (2002).

Homology modeling

A total of 28 3D structures were generated, 6 of which were modeled by homology with the Mcrα of *Methanosarcina barkeri* (1E6Y), and the target-template alignment showed 70% identity. The other 22 sequences were modeled by homology with the Mcrα of *Methanopyrus klanderi* (1E6V), showing an identity of 68%-70%. The 1E6Y and 1E6V template models mentioned above are available from the PDB. The 28 structures returned RMS values between 0.06 and 0.48 (see Supplementary Material, Table S1).

Clades 1 and 2 of the gene trees showed higher homology level with the Mcrα of *M. klanderi*. Figure 4 clearly shows their close similarity by the multiple alignment of target sequences, clades 1 and 2, and the template sequence, *M. klanderi*. Clade 3 and Tuc29 showed higher homology level with the Mcrα of *M. barkery*.

Ramachandran graphs were plotted for the evaluation of the stereochemical quality of the 3D structures (see Supplementary Material, Figure S1). These plots represent the distribution of the Φ and ψ angles in each residual of the aminoacid. Between 84.7 and 86.0% of the residuals of the 28 structures were located in favorable regions. The *z*-scores for residual energy varied between -5.66 and -5.92 (see Supplementary Material, Figure S2).

The 3D structures of each clade were superimposed. No significant differences were observed in the folding of the 3D structures when they were superimposed within the same clade. Based on this procedure, representative struc-
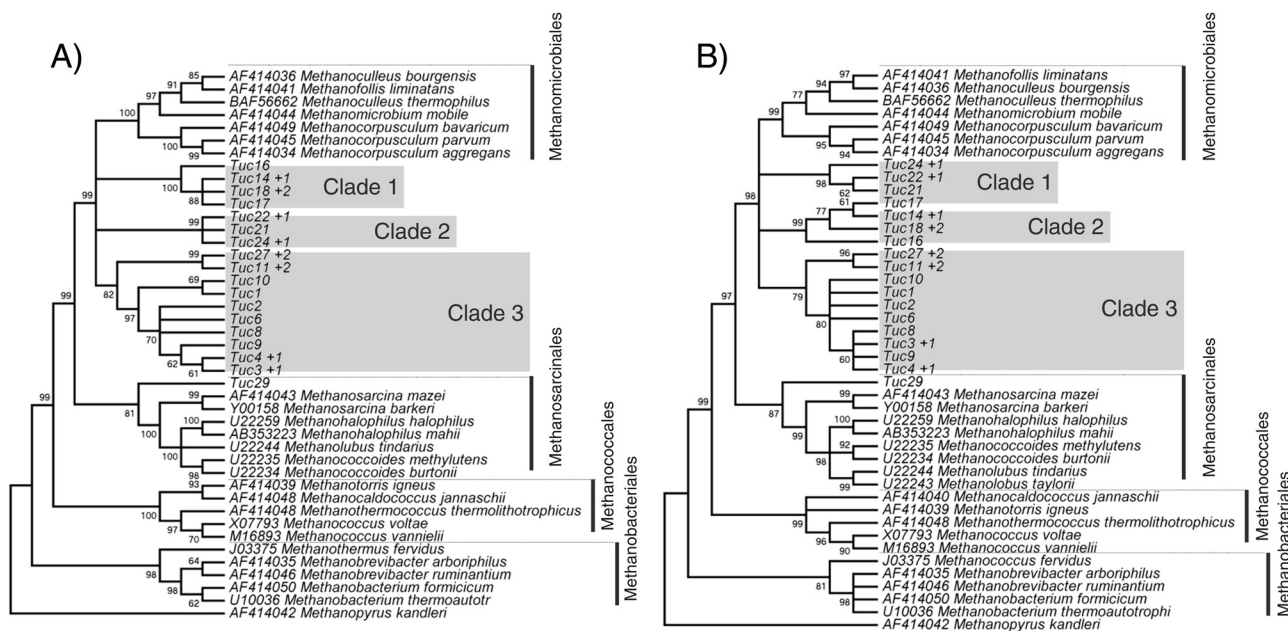

Figure 3 - Comparison of the gene trees for nucleotides (A) and amino acids (B). The sequences of the organisms identified by their scientific names were obtained from GenBank® (access numbers AF414034-AF414051), while those denominated "Tuc (number)" were identified in the present study (Genbank® accession number FJ715506 - FJ715617). The values +1 or +2 indicate the number of additional sequences equivalent to that shown (according to the Dotur program), and omitted from the tree.
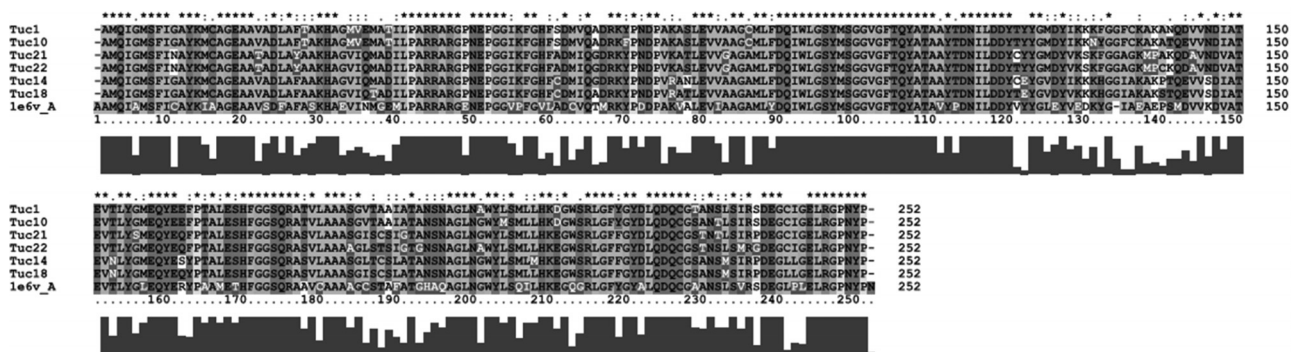
Figure 4 - Multiple alignment of the amino acid sequences of the Mcrα of *Methanospyrus klandery*, showing the template and the sequences obtained in the present study using the Clustal 2.0.9 program: Tuc14 and Tuc18 (clade 1), Tuc21 and Tuc22 (clade 2), and Tuc10 and Tuc1 (clade 3). Residuals identical to the sequence template are marked with an asterisk, and those that differ between the template and the target are marked with dots. The bars represent the degree of conservation of the respective residual.

tures were randomly selected from each clade for the comparison of the folding pattern among different clades. Once again, however, no significant difference was found between structures.

Discussion

The Mcr protein is made up of three subunits (α, β, and γ), which are each formed by two chains, where the α subunit, is the most popular for the inference of phylogenetic relationships (Hales *et al.*, 1996; Luton *et al.*, 2002; Hallan *et al.*, 2003; Castro *et al.*, 2004; Springer *et al.*, 2005; Juottonen *et al.*, 2006). Springer *et al.* (1995) and Luton *et al.* (2002) recorded broad similarities among the topologies of the arrangements obtained for 16S rRNA and the partial sequences of the *mcrα* gene.

Preliminary studies of the Tucuruí ecogenome (Graças *et al.*, personal communication) have shown, through the analysis of the 16S rRNA gene, the predominance of the order Methanomicrobiales in region C1 of UHE Tucuruí. The present analysis of *mcrα* corroborated these findings in 16S, and indicated an abundance of organisms closely related to the Methanomicrobiales. This order encompasses 24 recognized species in nine genera and three families, although the phylogeny of this group is extremely complex, and still not fully understood (Cavicchiolli, 2007).

The first rarefaction curve was based on the sequences obtained using the primers of Hales *et al.* (1996). The curve tended to plateau, suggesting that the number of unique OTUs is a good representation of the diversity of the archaean community of the C1 region of the Tucuruí reservoir. However, the second rarefaction curve which was based on the addition of the OTUs obtained using the primers of Luton *et al.* (2002), revealed a much higher diversity in comparison with the first curve. This indicates that, when only one pair of primers is used, the preferential amplification of some organisms may result in the underestimation of the methanogenic biodiversity of an area such as C1. The addition of the OTUs derived from the primers according to

Luton *et al.* widened the spectrum of organisms identified, and revealed a much higher diversity.

The surveys of Earl *et al.* (2003) and Castro *et al.* (2004) in the Priest Pot nature reserve in the UK and the WCA-2A conservation area in the northern Florida Everglades, (respectively) also found a predominance of methanogenic archaeans phylogenetically related to the Methanomicrobiales. These authors justified the pattern as the result of the higher temperatures and greater solar radiation during the summer months, which resulted in an increase in the microbian metabolism and anaerobic fermentation of organic material. In this case, the alterations in the biochemical composition of the water would have favored hydrogenotrophic methanogenesis, which is the preferential mode of biomethanogenesis in the Methanomicrobiales (Cavicchiolli, 2007). This same hypothesis may apply to region C1 of the Tucuruí reservoir, given that the area is characterized by relatively high temperatures and levels of solar radiation, as well as abundant organic material, throughout the year. An additional factor favoring the production of hydrocarbons is the fact that the region is anoxic.

Methanogenesis is carried out through the syntrophic association of micro-organisms capable of fermenting glucose into shorter-chain carbon compounds such as formate, acetate, methanol, methylamines, methylthios and CO_2 (Thauer, 1998). This process can have three distinct routes: hydrogenotrophic (Horn *et al.*, 2003), acetylclastic or methylotrophic (Ferry, 1999). These pathways vary in the preferential use of different substrates for the acquisition of metabolic energy. In general, the three routes converge in a sequence of seven reactions, the last of which is induced by the enzyme Mcr, which catalyzes the synthesis of heterodisulfide (CoM-S-S-CoB) from the substrates coenzyme M (CH_3-S-CoM) and coenzyme B (H-S-CoB), liberating methane (CH_4) as a subproduct (Thauer, 1998):

$$CH_3\text{-S-CoM} + \text{H-S-CoB} \quad \text{CoM-S-S-CoB} + CH_4$$

Little is known of the 3D structure of Mcrα. Crystallographic Mcr could only be found in the PDB for three

methanogenic archaeans - *M. klanderi* (Grabarse *et al.*, 2000), *M. barkeri* (Grabarse *et al.*, 2000), and *Methanobacterium thermoautotrophicum* (Ermler *et al.*, 1997). In the present study, the Mcrα of the first two of these organisms were selected as the template for homology modeling. Despite the apparent phylogenetic distance between *M. klanderi* and *M. barkeri*, the template and target were highly homologous.

For the analysis of 3D structure, the three subgroups of the gene tree were treated separately. The RMS values for the 28 structures obtained varied from 0.06 to 0.48, which indicates only a slight deviation between targets and the templates, supported by their identity values (68%-70%). No significant differences were observed in the folding of the 3D structures when target and templates of the same clade were superimposed. Given this, representative structures of each clade were selected randomly for the comparisons among the different clades, which indicated that the 3D structures of the Mcrα of archaeans of different orders tend to be well conserved (Fong *et al.*, 2007), despite their characteristic differences in the distribution of amino acid residues on the protein surface (Grabarse *et al.*, 2000).

The Ramachandran graphs indicated that all 28 targets structures analyzed were of good stereochemical quality, given that 85 to 86% of the residuals of the amino acids modeled presented spatially viable angles, being located in favorable areas of the graph, where the angles and residual energy are compatible (Morris *et al.*, 1992). The variety of possible random protein configurations and the respective deviation of the total structures's energy are shown by z-score graphs (Sippl, 1993). These scores varied between -5,66 and -5,92 for the 28 targets obtained from the homology modeling, which indicates that the structures generated are energetically stable. The scarcity of crystallographic models of Mcr for the archaea of the order Methanomicrobiales did not limit the effectiveness of the homology modeling, given the high identity scores (68%-70%) recorded between the template and target structures, subsequently confirmed by the generation of structures with high indices of quality.

The nucleotide sequence of the alpha subunit of the *mcr* gene and the reduced number of unique OTUs indicates the existence of a relatively low diversity of methanogenic archaea (83%-95% identity with the NCBI data bank) in region C1 of the ecogenome of the Tucuruí reservoir. The *mcr*α gene has proven to be a useful tool for the study of diversity in methanogenic archaeans, especially considering its ample distribution among these organisms. In the archaeans of the C1 region of the Tucuruí reservoir low levels of polymorphism in nucleotides, predicted amino acids, and Mcrα 3D structures. The majority of the mutations observed in the nucleotide sequences did not cause any changes in the residuals, and where this did occur, the substitutions modified the 3D structure of the *mcr* alpha fragment only slightly.

The present study is the first of its kind, to our knowledge, to investigate the ecology of the methanogenic archaea of a tropical hydroelectric reservoir, and contribute to the understanding of the principal organisms responsible for biomethanogenesis in the aquatic environments of the Amazon basin. The study also demonstrated the usefulness of the comparative modeling of proteins as a tool for the elucidation of the 3D structure of the Mcr protein alpha subunit, and that this constitutes a promising approach for the understanding of the equilibrium between methanogenesis and methanotrophy in Amazonian hydroelectric reservoirs.

Acknowledgments

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Supplementary Material

The following online material is available for this article:

Table S1 - RMS values and percentage of identity between the model e target structures.
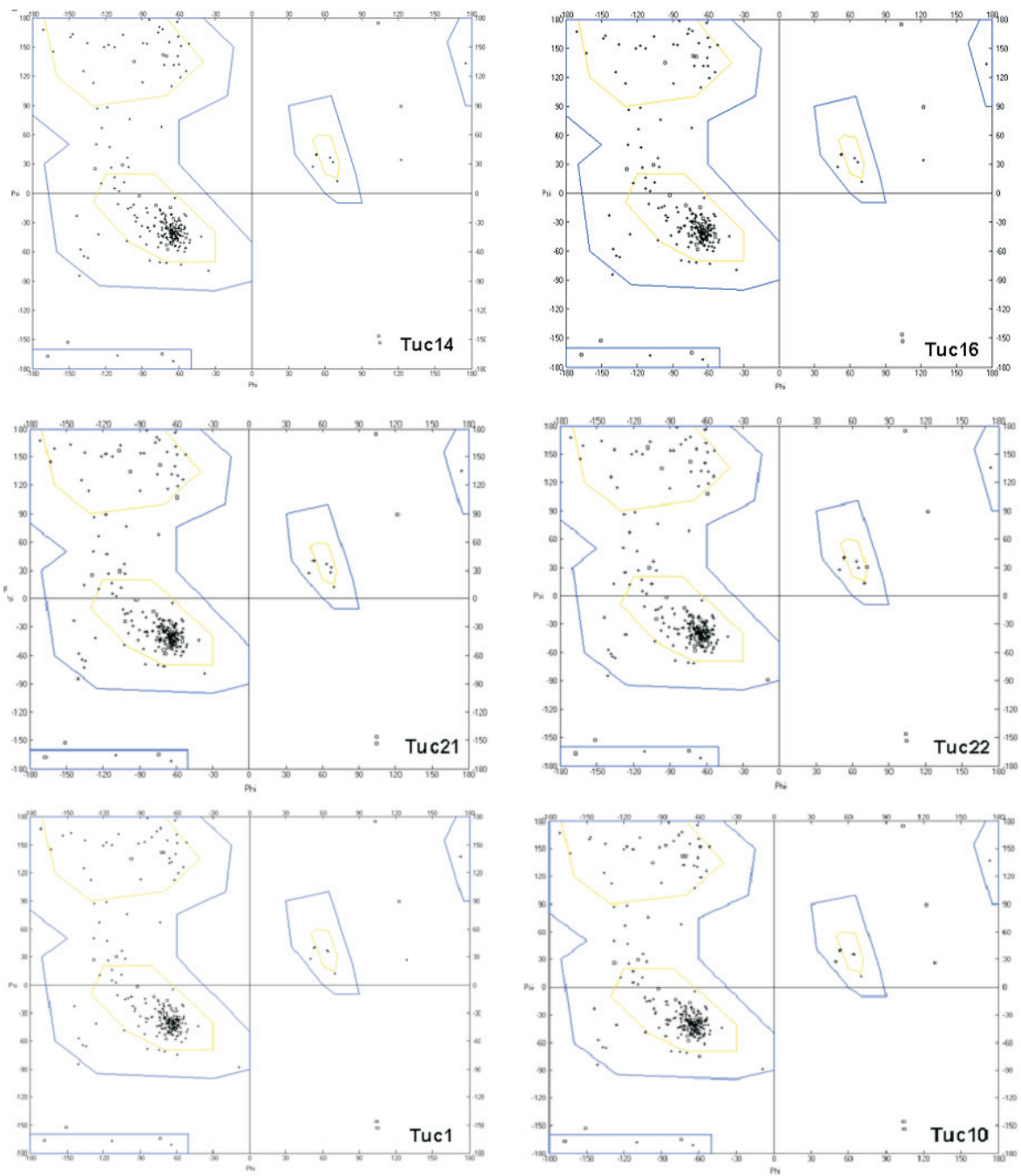
Figure S1 - Ramachandran plots of the 3D structures.

Figure S2 - The z-scores plots for the 3D structures.

This material is available as part of the online article from http://www.scielo.br/gmb.

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Table S1 - RMS values and percentage of identity between the model and target structures.

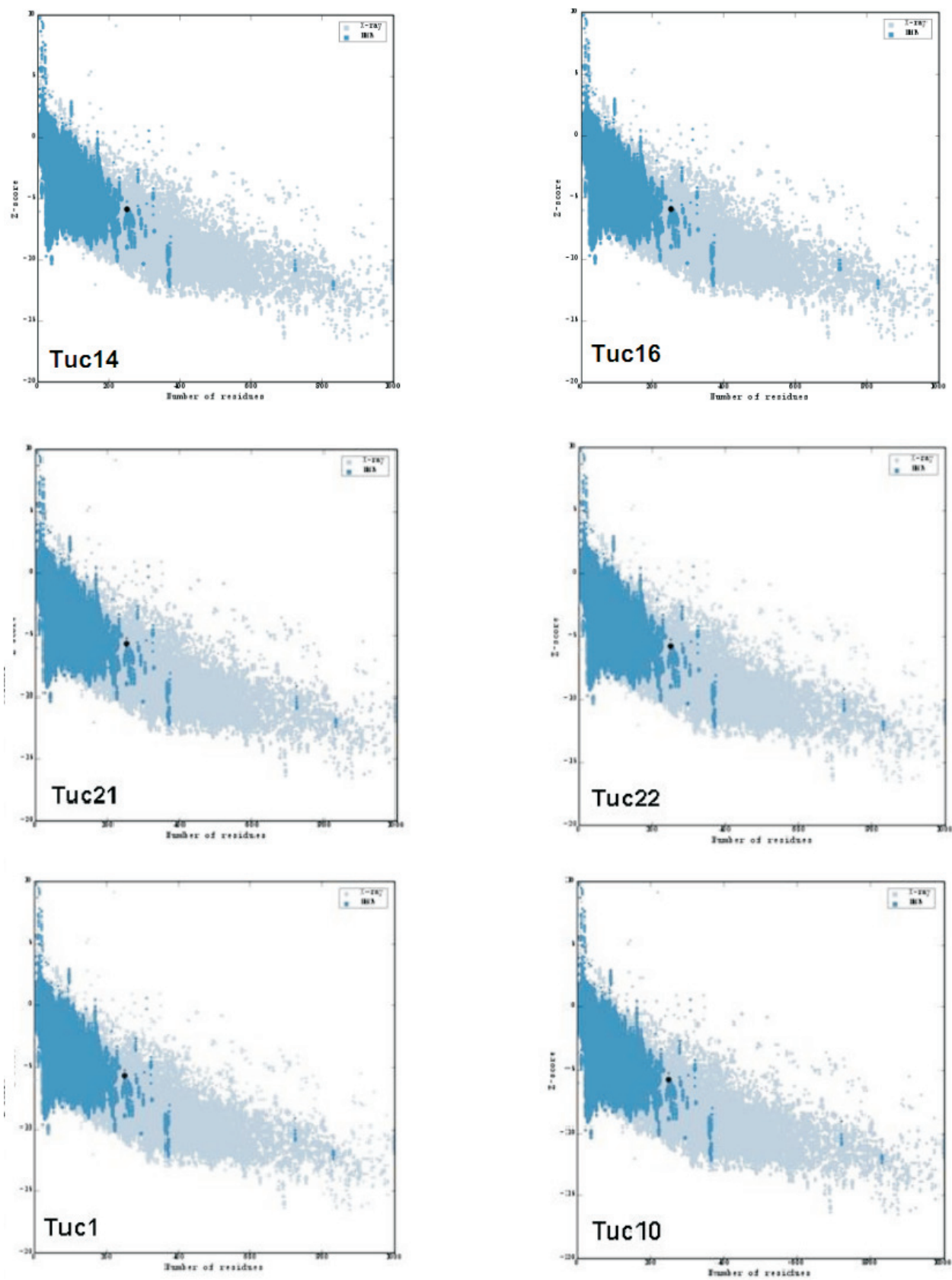| 3D Structures	RMS	Identity
Tuc1	0.064	69%
Tuc2	0.065	70%
Tuc3	0.489	70%
Tuc4	0.067	69%
Tuc5	0.067	69%
Tuc6	0.490	70%
Tuc7	0.065	68%
Tuc8	0.066	69%
Tuc9	0.066	68%
Tuc10	0.067	68%
Tuc11	0.064	68%
Tuc12	0.065	68%
Tuc13	0.065	68%
Tuc14	0.064	69%
Tuc15	0.067	68%
Tuc16	0.065	69%
Tuc17	0.064	68%
Tuc18	0.064	69%
Tuc19	0.064	69%
Tuc20	0.064	69%
Tuc21	0.068	68%
Tuc22	0.066	70%
Tuc23	0.067	70%
Tuc24	0.065	70%
Tuc25	0.486	70%
Tuc26	0.486	70%
Tuc27	0.486	69%
Tuc29	0.067	71%

Figure S1 - Ramachandran plots of the 3D structures.

Figure S2 - The z-scores plots for the 3D structures.