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Identification of α-L-fucosidase (ALFuc) of *Blastocystis* sp. subtypes ST1, ST2 and ST3

Joel Martínez-Ocaña[1], Williams Arony Martínez-Flores[1], Angélica Olivo-Díaz[1], Mirza Romero-Valdovinos[1], Fernando Martínez-Hernández[1], Guillermo Aguilar-Osorio[2], Ana Flisser[3], Pablo Maravilla[1]

ABSTRACT

Blastocystis sp. is a common intestinal microorganism. The α-L-fucosidase (ALFuc) is an enzyme long associated with the colonization of the gut microbiota. However, this enzyme has not been experimentally identified in *Blastocystis* cultures. The objective of the present study was to identify ALFuc in supernatants of axenic cultures of *Blastocystis* subtype (ST)1 ATCC-50177 and ATCC-50610 and to compare predicted ALFuc proteins of *alfuc* genes in sequenced STs1–3 isolates in human *Blastocystis* carriers. Excretion/secretion (Es/p) and cell lysate proteins were obtained by processing *Blastocystis* ATCC cultures and submitting them to SDS–PAGE and immunoblotting. In addition, 18 fecal samples from symptomatic *Blastocystis* human carriers were analyzed by sequencing of amplification products for subtyping. A complete identification of the *alfuc* gene and phylogenetic analysis were performed. Immunoblotting showed that the amplified band corresponding to ALFuc (~51 kDa) was recognized only in the ES/p. Furthermore, prediction analysis of ALFuc 3D structures revealed that the domain α-L-fucosidase and the GH29 family's catalytic sites were conserved; interestingly, the galactose-binding domain was recognized only in ST1 and ST2. The phylogenetic inferences of ALFuc showed that STs1–3 were clearly identifiable and grouped into specific clusters. Our results show, for the first time through experimental data that ALFuc is a secretion product of *Blastocystis* sp., which could have a relevant role during intestinal colonization; however, further studies are required to clarify this condition. Furthermore, the *alfuc* gene is a promising candidate for a phylogenetic marker, as it shows a conserved classification with the *SSU-rDNA* gene.

KEYWORDS: α-L-fucosidase. *Blastocystis* sp. Glycoside hydrolase. Phylogenetic marker. Subtypes.

INTRODUCTION

Blastocystis is an anaerobic stramenopile that colonizes the intestinal tract of several taxa[1]. Previous reports focused on the speciation of isolates was based on host species. However, a subtype (ST) classification system based on *SSUr-DNA* genes was established whereas molecular typing revealed a disparity in host-based classification[2]. In addition, guidelines were proposed to correctly identify new *Blastocystis* STs to avoid confusion in the literature; at present, 25 subtypes meet the currently recommended criteria for unique subtype designations (STs1–17, ST21, STs23–26, and STs27–29), while STs18–20 and ST22 have been considered insufficient[2,3]. Although their pathogenicity in humans remains controversial, more than one billion *Blastocystis* carriers could exist worldwide[4]. STs 1–3 have been reported as the most

[1]Hospital General "Dr. Manuel Gea Gonzalez", Departamento de Ecología de Agentes Patógenos, Ciudad de México, México

[2]Universidad Autonoma de México, Instituto de Química, Ciudad de México, México

[3]Universidad Autonoma de México, Facultad de Medicina, Departamento de Microbiologia y Parasitologia, Ciudad de México, México

Correspondence to: Williams Arony Martínez-Flores
Hospital General "Dr. Manuel Gea Gonzalez", Departamento de Ecología de Agentes Patógenos, Calzada de Tlalpan, 4800, Colonia, Sección XVI, 14080, Ciudad de México, México

E-mail: Williams.arony@salud.gob.mx

Pablo Maravilla
Hospital General "Dr. Manuel Gea Gonzalez", Departamento de Ecología de Agentes Patógenos, Calzada de Tlalpan, 4800, Colonia, Sección XVI, 14080, Ciudad de México, México

E-mail: maravillap@yahoo.com

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prevalent in human populations, however, other STs have also been found in humans and animals (ST4–10, ST12, ST14, and ST16). On the other hand, some STs can infect birds and mammals[4], however, isolates from amphibian and reptiles appear to be restricted to these groups[5,6].

Intestinal microorganisms can express/secrete different molecules that interact with the host intestinal mucosa, among them, the α-L-fucosidase (ALFuc), a glycoside hydrolase (GH)[7]. According to the "Carbohydrate-Active EnZYmes Database" n.d. (CAZy), ALFuc is an enzyme that catalyzes the hydrolytic removal of L-fucose residues that bind to the non-reducing end of glycan chains, such as mucins[8]. According to their amino acid sequence, α-L-fucosidases are classified into two families: GH29 and GH95. GH29 enzymes are a broad family of retention fucosidases active on α*(1,2)-*, α*(1,3)-*, α*(1,4)-*, and α*(1,6)-L-fucosyl* ligands. GH29 has been further divided into subfamilies A and B, with GH29A being active on a wide range of ligands, whereas GH29B is specific for α*(1,3)-* and α*(1,4)-L-fucosyl* ligands. GH95 enzymes are a small family of inverted fucosidases active on α*(1,2)-fucosyl galactose*[9].

A significant link has been established between human α*-L-fucosidase* (termed FUCA2) and *Helycobacter pylori* adhesion, growth and pathogenicity[10]. Furthermore, it has been documented that growth and invasion of *Campylobacter jejuni fuc*+, 129, 108, and NCTC 11168 strains are increased in the presence of active L-fucosidases released by *Bacteriodes fragilis*, demonstrating that *C. jejuni* is dependent on external fucosidases for further growth and invasion[11]. It has also been shown that the ability of bifidobacteria to metabolize fucosylated compounds (found in breast milk) via fucosidases is an essential mechanism for shaping the intestinal microbiome in humans during the first months of life[12].

There is plenty of information on ALFuc in bacteria. However, ALFuc has not been experimentally identified in *Blastocystis* cultures. Therefore, the objective of the present study was to identify ALFuc in supernatants from commercial axenic cultures of *Blastocystis* ST1, and to compare predicted ALFuc proteins of *alfuc* genes after sequencing STs1–3 isolates from human *Blastocystis* carriers.

MATERIALS AND METHODS

Ethics statement

The current study was approved by the Research and Ethics Committee of the "Dr. Manuel Gea Gonzalez" General Hospital, with reference number 12-77-2018. Written consent was obtained from all participants.

Blastocystis in vitro cultures

The axenic commercial *Blastocystis* ST1 cultures ATCC-50177 and ATCC-50610 were obtained from The American Type Culture Collection (ATCC) and used in this study to perform a protein analysis. For the propagation of axenic strains of *Blastocystis* ST1 ATCC 50177 and 50610, the data sheets recommend the use of *Blastocystis* egg biphasic medium ATCC 1671 supplemented with 10% of horse serum. The constitution of the medium is as follows: 130 mM NaCl, 1 mM $CaCl_2$, 2 mM KCl, 0.4 mM $MgCl_2$, 10 mM Na_2HPO_4, 4 mM $NaHCO_3$, and 2 mM KH_2PO_4. For its preparation, egg yolks were emulsified and sieved; 4 mL were deposited per tube, then eggs were solidified at 60 °C by placing the tubes in an inclined position. Subsequently, 4 mL of Stone's modified Locke's solution were added. The tubes were then sterilized. At the time of use, 10% of horse serum was added and 1 mL of the suspension of each axenic strain of *Blastocystis* was deposited. Cultures were kept in an anaerobic jar with BD GasPak (BD, Franklin Lakes, NJ, USA) for 48 h at 37 °C. After that, they were inoculated into 10 mL of Iscove's modified Dulbecco's medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% of inactivated horse serum (PAA Laboratories GmbH, Pasching, Austria) and 1% of penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The cultures were grown in an anaerobic flask (Merk Millipore, Burlington, MA, USA), containing a BD GasPak (BD, Franklin Lakes, NJ, USA) and incubated at 37 °C for 48 h[13]. Afterward, microscopic observations were performed only on some cultures to confirm cellular growth.

Eighteen fecal samples from adult *Blastocystis* carriers who attended medical consultation at the "Dr. Manuel Gea Gonzalez" General Hospital due to gastrointestinal disorders were screened by microscopy and xenic isolation procedures were carried out within 6 h of deposition[13]. Pea-sized pieces of stool or 250 µL liquid samples were inoculated into 8 mL of Jone's medium supplemented with 5% of inactivated horse serum[13] (PAA Laboratories GmbH, Pasching, Austria) using six culture tubes per sample. The culture tubes were incubated at 37 °C for 48 h (during exponential growth), and the success of the isolation was confirmed using microscopy.

Preparation of *Blastocystis* protein extracts and culture supernatants

For the excretion/secretion protein (Es/p) analysis, ATCC-50177 and ATCC-50610 cultures were centrifuged at 6,000 x g for 10 min, and supernatants (~15 mL)

and cellular pellets (~850 *Blastocystis* cells/mL using a Neubauer chamber) were separately recovered. Then, the supernatants were centrifuged at 16,000 x g at 4 °C for 10 min and passed through a 0.20 µm filter (Corning, Merk Millipore, Burlington, MA, USA) before being placed in an Amicon Ultra-15 100,000 NMWL (Merk Millipore, Burlington, MA, USA) and centrifuged at 4,000 x g for 40 min at 4 °C to remove proteins with a molecular weight greater than 100 kDa. Finally, ES/p was concentrated for 40 min at 4 °C using an Amicon Ultra-4 10,000 NMWL at 4,000 x g, and proteins were resuspended in 2 mL of 50 mM Tris-HCl pH 7.5 and stored at -70 °C until use[14].

For the analysis of *Blastocystis* cell lysate (Bcl), the previously obtained cellular pellets were washed three times with phosphate-buffered saline (PBS) 1X pH 7.2 and centrifuged at 6,000 x g for 10 min. The parasites were lysed by ten freeze-thaw cycles at -70 °C and 37 °C, sonicated on ice using ten one-minute pulses, separated by one minute of relaxation, divided into aliquots, and stored at -70 °C until required[13]. For both ES/p and Bcl, the total protein concentration was determined by a Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA)[15].

Identification of *Blastocystis* ALFuc.

The annotated α-L-fucosidase protein for *Blastocystis* ST7 [UniProtKB-D8M3D3] available in the UniProt database[16] was used to predict its theoretical molecular mass with ExPASY-Compute pI/Mw[17] for further comparisons. After standardizing and testing different concentrations of Es/p and Bcl, the optimal technical conditions were established, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to identify the putative ALFuc; to this end 60 µg of Bcl and Es/p were loaded in 2x loading buffer containing 3% of SDS, 150 mM Tris-HCl, pH 6.8, 30% glycerol and 0.1% bromophenol blue. Samples were placed in boiling water for 5 min and then on precast 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories, Hercules, CA, USA) at 100 volts/h. Coomassie brilliant blue staining was used to identify the putative ALFuc protein band and calculate its Rf using Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA). Three electrophoretic runs were used to perform the relative mobility (Rf) calculation[18].

Immunoblotting for ALFuc

To perform electrophoresis and then transfer the proteins, 20 mg of Es/p and Bcl each were analyzed using the same conditions described for SDS–PAGE. Then the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Merk Millipore, Burlington, MA, USA) at 100 volts/h. The presence of protein bands was verified by using Ponceau red staining. To standardize the immunoblot conditions, several assays were performed, testing different concentrations of antibodies as well as blocking agents, until the optimal conditions were found. Nonspecific binding sites were blocked in the membrane by incubating it with 1X Blocking Reagent (Roche, Merk Millipore, Burlington, MA, USA) in 50 mM Tris-Base pH 7.5, 0.15 M NaCl, 0.03% Tween-20 (TBS-T) for 1h at room temperature, followed by three TBS-T washes and overnight incubation with anti-α-L-fucosidase (Santa Cruz Biotechnology, Dallas, TX, USA) at a 1:2,000 dilution in blocking buffer with 0.03% TBS-T at 4 °C. The membrane was then washed three times with TBS-T and incubated with biotinylated goat anti-mouse anti-IgG (Santa Cruz Biotechnology, Dallas, TX, USA) at 1:4,000 dilutions for 2 h at room temperature. After three washes, the membrane was incubated with streptavidin-peroxidase (Jackson Immunoresearch, West Grove, PA, USA) at a dilution of 1:10,000 for 1 h at room temperature, followed by three washes with TBS-T. Finally, the membrane was treated with diaminobenzidine (DAB) (Sigma-Aldrich, Merk Millipore, Burlington, MA, USA)[19].

DNA extraction and PCR for the *alfuc* gene

One milliliter of each of the 18 fecal samples, ATCC-50177 and ATCC-50610 cultures, was recovered for DNA extractions. A QIAmp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used following the manufacturer's instructions to extract the DNA from the *Blastocystis* pellets. The DNA samples were eluted in 50 µL of elution buffer and stored at -70 °C. The extracted DNA was used for polymerase chain reactions (PCR) to obtain a partial sequence of the *SSUrDNA* gene[20] to identify the *Blastocystis* STs in xenic cultures, according to current guidelines[13]. Four new primer pairs were designed in this study to amplify four overlapping regions and to assemble a sequence of approximately 2,500 base pairs (bp) (Table 1), which included the whole *alfuc* gene for STs1–3 and partial regions of the eIF-3 translation initiation factor subunit 4 (*eif3S4*) and the estradiol 17-beta-dehydrogenase (*hsd17B*), as well as two flanking intergenic regions (accession Nº LXWW01000289.1, region: 82284-84859)[21]. In addition to the two strains ATCC 50177 and 50610, a PCR was performed with eighteen isolates obtained from patients to amplify the complete sequence of the *alfuc* gene, using 25 µL volumes: 1X PCR buffer, 2.4 mM $MgCl_2$, 0.5 mM dNTPs, 0.01 mg BSA, 1 U Taq DNA Polymerase

Table 1 - Primers used to amplify partial regions of the *SSUr-RNA* gene and the *alfuc* gene of *Blastocystis*.

Locus	Primer name	Position[$]	Size[¥] (bp)	Direction	5'-3' sequence
SSU-rRNA[&]	Blast 505-532	637-1411	~492	Forward	GGAGGTAGTGACAATAAATC
	Blast 998-1017			Reverse	TGCTTTCGCACTTGTTCATC
α-L-fucosidase[£]	ALFUC/S1	1-928	~872	Forward	AC**W**CCCTCGTTTCC**W**CC**MKY**AG
	ALFUC/As1			Reverse	TTCAG**Y**TTCAC**R**GGGTTGAAGAT
	ALFUC/S2	767-1639	~860	Forward	GAGGAGCA**R**G**YY**CA**R**TGGCT
	ALFUC/As2			Reverse	GTGGTAGAACCAGCC**S**GG**K**C
	ALFUC/S3	1240-2093	~850	Forward	TGGAGACCGGAGTACAACGA
	ALFUC/As3			Reverse	TCTT**S**AC**R**CC**R**ATGGTGGT**K**CCC
	ALFUC/S4	1971-2678	~675	Forward	TCAACGTG**RTS**ATGATGCAGGA
	ALFUC/As4			Reverse	TCC**R**TC**VSTK**GC**W**CC**Y**GT**S**ACC

& = Primer set as described by Santin *et al.*[20] to detect and subtype *Blastocystis* of human and animal origin; £ = Primer set designed in this study to amplify four overlapping regions from partial eif3S4, complete *alfuc* and partial hsd17B of *Blastocystis* STs1–3; $ = Positions of the sequenced regions in the alignments of the complete *SSU-rRNA* gene of *Blastocystis* STs1–17 and non-human/other mammal/bird sources; positions of the four overlapping regions according to sequence alignments of ST1 genomic regions [accession number: LXWW01000289, region: 82284..84859], ST2 [accession number: JZRJ01000159, region: c8456..10980] and ST3 [accession number: JZRK01000455, region: 1512..3972]; ¥ = Approximate expected amplification sequence size on different loci. Bold nucleotides in the primer sequences indicate degenerated nucleotides to anneal targeted positions of STs1–3.

(Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 2-5 µL of DNA and nuclease-free water. The PCR products were confirmed by 1% agarose gel electrophoresis. For each *alfuc* region, 200 µL of PCR products were purified using the AxyPrep PCR Clean-up kit (Axigen Biosciences, Union City, CA, USA) following the manufacturer's instructions. The sequences were obtained using the Sanger method at the National Biodiversity Laboratory, Institute of Biology of the Universidad Nacional Autonoma de Mexico (NBL-BI-UNAM).

Three-dimensional structure prediction

The I-TASSER[22] server was used to model the 3D structure of *Blastocystis* ALFuc corresponding to ST1, ST2, and ST3 obtained by translation of the complete *alfuc* gene. This server is under active development intending to provide the most accurate predictions of protein structure and function using state-of-the-art algorithms. After analysis, the models with the highest confidence scores (C-score) were selected. The three-dimensional structures were then analyzed and visualized using PyMOL 2.5.2[23].

Phylogenetic analysis

The quality of chromatograms of the sequenced regions was evaluated with the programs phred, phrap and consed, versions 0.11220.c, 1.090518 and 29, respectively (Seattle, WA, USA). The assembly of the *alfuc* gene sequences was guided using the genomic regions containing putative *eif3S4-alfuc-hsd17B* for STs1–3 as a reference. In addition, we used two sequence datasets from *Blastocystis* STs1–3 and STs4–9 for the validated SSUr-RNA and the predicted alpha-L-fucosidase genes and proteins (Supplementary Table S1). *Proteromonas lacertae* [SSU-rRNA accession Nº U37108.1[21] and predicted *alfuc* accession Nº NGBS01001212[21] region: c18708.20188] was used as an outgroup. Multiple sequence alignments were computed in ClustalW version 2.0 and trimmed with trimAl version 1.2 as implemented in Phylemon version 2.0. ModelTest-NG version 0.2.0 was used to select the best-fit substitution model for nucleotide alignments (SSUr-DNA: HKY+G and *alfuc* gene: K2+G). According to the common methods of phylogenetic inference, maximum likelihood trees were constructed in MEGA X software version 10.1.8 with 1,000 bootstrap replicates. Bayesian trees were computed in Mr. Bayes Software version 3.2.6 for four million generations; the posterior probability distribution and diagnostic frequency were sampled every 1,000 and 105 generations, respectively; and a substitution model was implemented during the analysis by reversible jump. The trees were summarized with a post-burning sample of 50%. Figtree version 1.4.4 was used to edit the phylogenetic trees. The *alfuc* gene sequences were annotated by comparison with the genomic regions (Supplementary Table S1) that code for *Blastocystis* ALFuc sequences, available for ST1 [accession Nº OAO14080.1][24], ST4 [accession Nº XP_014526040.1][24] and ST7 [accession Nº CBK22406.2][24]. The predicted amino acid sequences were further analyzed to theoretically calculate both, the molecular mass and the

isoelectric point with ExPASY-Compute pI/Mw software[17] to predict the signal peptides with SignalP-5 and to identify the conserved domains in the Pfam database[25].

RESULTS

Identification of ALFuc from *Blastocystis* axenic cultures

To identify the ALFuc of *Blastocystis* in SDS-PAGE, the theoretical molecular mass of 51.5 kDa was calculated from the sequence annotated for α-L-fucosidase of *Blastocystis* ST7 in GenBank, using ExPASY-Compute pI/Mw. To obtain this theoretical result, a strong protein band that migrated ~50 kDa was identified in the electrophoresis by staining with Coomassie blue, both in Bcl and in Es/p in the two strains 50177 and 50610 analyzed (Figure 1A). The molecular mass of this protein was calculated by plotting a graph of Rf vs log apparent molecular mass and interpolating the value of Rf = 0.52, obtaining the calculated molecular mass of 51 kDa (Supplementary Figure S1A). This result was consistent with the theoretical molecular mass initially calculated by ExPASY-Compute pI/Mw. In addition, the immunoblot for ALFuc showed a clear recognition of the commercial antibody[26] against this same protein band of ~51 kDa in Es/p of both 50177 and 50610 strains, and to a much lesser extent in their Bcl (Figure 1B).

Comparison of *alfuc* gene sequences in ST1, ST2 and ST3.

To evaluate the variability of the *alfuc* gene in the most common *Blastocystis* STs in humans, subtyping and phylogenetic inferences were performed. The most common STs identified within the 18 isolates were ST1 (3/18), ST2 (7/18), and ST3 (8/18) were confirmed by comparing their partial *SSUrDNA* sequences with a sequence dataset of validated subtypes (Figure 2). The sequenced genomic region containing the *alfuc* gene was obtained for each of the 18 xenic isolates and the two axenic strains. The sequence was then annotated as described above and then compared to the homologous regions in STs1–4 and STs6–9 to define putative exonic and intronic regions in the *alfuc* gene, trim the intergenic regions and detect *eif3S4* and *hsd17B* partial genes. These comparisons recognized the existence of two putative ORFs (open reading frames): ORF1 based on ST4 annotation, identified in all STs (STs1–4 and STs6–9), and ORF2 based on ST1 annotation, identified only in ST1 and ST8. The primary variation between the two ORFs was that ORF2 starts at intron one and can be translated into a shorter protein without a signal peptide (Supplementary Table S1). These proteins were analyzed using Pfam[25] to identify the presence of conserved domains in the proteins obtained in this study through amplification of the complete *alfuc* gene and its subsequent translation into amino acids.

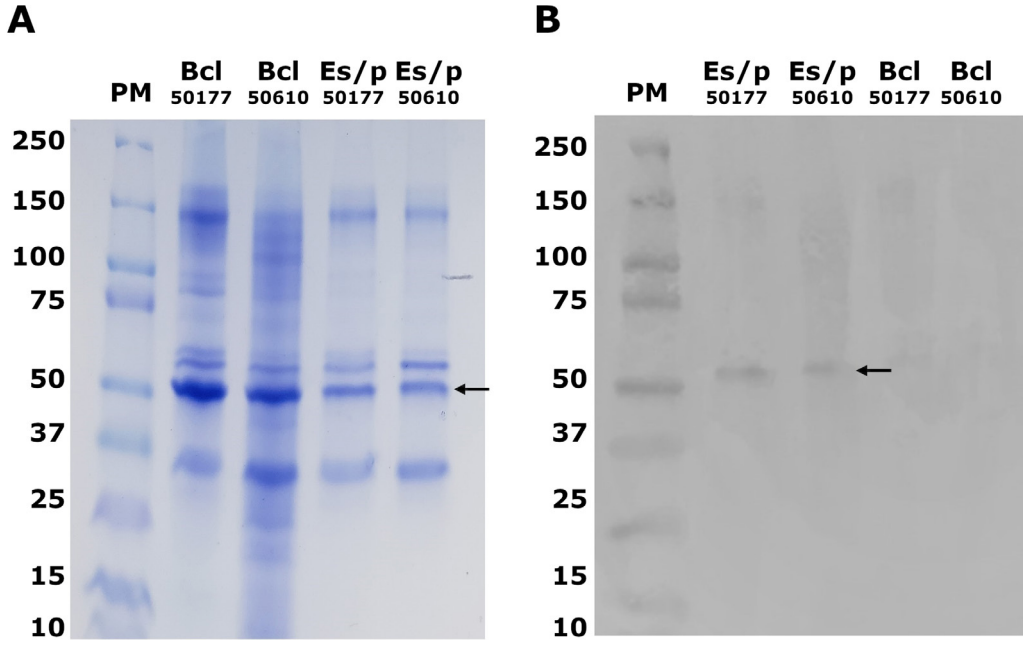

Figure 1 - SDS-PAGE and Immunoblot of ALFuc *Blastocystis*: A) SDS-PAGE, stained with Coomassie blue of cells lysate (Bcl) and *Blastocystis* excretion/secretion proteins (Es/p), from axenic strains ATCC 50177 and ATCC 50610 are shown. The arrow indicates the molecular mass calculated by the electrophoretic mobility (Rf) of 51 kDa for *Blastocystis* ALFuc; B) the recognition of *Blastocystis* ALFuc by a commercial anti-α-L-fucosidase antibody is observed. The arrow indicates the protein recognized by the antibody with an expected molecular weight of 51 kDa which is consistent with the calculated molecular mass on SDS-PAGE for *Blastocystis* ALFuc.

According to this analysis, we identified the characteristic domain (IPR000933) of the GH29 family of glycoside hydrolases, the conserved domain of the α-L-fucosidase family of the CL0058 clan, and a similar galactose-binding domain (IPR008979), identified as the C-terminal domain (IPR000421) of coagulation factors 5/8 (Supplementary Figure S1B). In addition, in these same 13 proteins with ORF1, the signal peptide was identified at the N-terminal end. In proteins with ORF2, no signal peptide was present. The SignalP-5.023[27] analysis identified the signal peptide through peptide excision only in sequences with ORF1. It was found between amino acids 16 and 17 (VLA-RP) with a probability of 0.6139 in ST1 and 0.79 in ST2, in amino acid positions 14–15 (ALA-KP) with a 0.79 probability for ST3, and in positions 15–16 (VLS-KR). Analysis for transmembrane regions showed negative results for all proteins.

To strengthen the result obtained by the Pfam analysis confirming that our proteins belong to the GH29 family of α-L-fucosidases, we performed a ClustalW alignment on the proteins with three α-L-fucosidases characterized as belonging to the GH29 family: *Bacteroides thetaiotaomicron* (*B. theta.*) [UniProtKB-Q8A085][16], *Bifidobacterium bifidum* [UniProtKB-C5NS94][16] and *Streptomyces sp*. [UniProtKB-Q9Z4I9][16]. Our alignment analysis showed a high conservation of the characteristic catalytic site of the α-L-fucosidases of the GH-29 family, consisting of the characteristic catalytic nucleophilic residue Asp (D) and the acid/base residue Glu (E) (Figure 3).

Prediction of three-dimensional structures

The designed structures were analyzed by the I-TASSER server. This server generates some structural conformations, then used the SPICKER program to group all the structures based on the similarity of their paired structures. Finally, for *Blastocystis* ALFuc ST1, ST2 and ST3 the server reported five main models each of them, corresponding to the five largest clusters. The confidence of each model was calculated using the C-score. The C-score values showed the accuracy of the predicted model, which is usually in the range of -5 to 2. In addition, the higher the value of the C-score, the better the quality of the prediction. The C scores of the *Blastocystis* ALFuc models ST1, ST2, and ST3 were 1.22, 0.93, and 0.83, respectively. Therefore, ALFuc *Blastocystis* ST1 with a C score of 1.22 showed greater accuracy among the predicted models. Figure 4 shows the tertiary structures of the ALFuc of *Blastocystis* ST1, ST2, and ST3. In the image, one can see the domain of the α-L-fucosidase, consisting of five α-helix structures (magenta color); the similar domain of binding to galactose, formed by five structures of β-strand (orange color). In ST3, the Pfam result for this domain was negative. Likewise, the catalytic site of the GH29 family was observed, formed by the nucleophilic residue D (red spheres) and the acid/base residue E (blue spheres).

Phylogenetic analysis

The phylogenetic relationships of *Blastocystis* were explored to determine whether α-L-fucosidase could infer the characteristic of the *SSU-rDNA* topology or the one described for *nad* genes from the *Blastocystis* mitochondrion-related organelle (MRO) genomes. Therefore, we undertook phylogenetic analyses using two partial regions and the complete *alfuc* genes from *Blastocystis* STs1–3 and STs4–9. Partial region two spans most of the α-L-fucosidase domain, and region three contains the galactose-binding-like domain. Our results confirmed a conserved identity in the tree topology among *alfuc* and SSU-rDNA in the phylogenetic analysis. The gene tree topology (Figure 3) is similar to that described for *Blastocystis* by SSU-rDNA[28], MRO *nad* genes[29], and Miro protein[30]. The topology consisted of ST1 and ST2 clustered together; ST3 clustered or associated with ST4 and ST8, and ST7 clustered or associated with ST6 and ST9 (Figure 5). Interestingly, in the phylogenetic analysis of the two partial regions of the *alfuc* gene, ST3 and ST7 shuffled their associations from their common clusters, while the STs1/2, STs4/8 and STs 6/9 clusters remained consistent (data not shown).

DISCUSSION

In the present study, ALFuc was identified in Es/p by predicting its molecular mass in electrophoresis, further using a commercial antibody directed against the secreted α-L-fucosidase, suggesting that this enzyme could be part of the secretion products of *Blastocystis*. This finding is consistent with previous reports predicting ALFuc as a secreted protein related to carbohydrate metabolism in *Blastocystis*[31], but this claim has not been supported by experimental evidence so far.

Likewise, through the amplification of the *alfuc* gene, and its subsequent translation into proteins, the amino acid sequences of the ALFucs ST1-3 were obtained. These sequences were analyzed using Pfam to identify the domains. The result of this analysis showed the identification of the characteristic domain of α-L-fucosidases, GH29 enzymes, and the similar galactose-binding domain (only in ST1 and ST2). To support these findings, the catalytic sites of

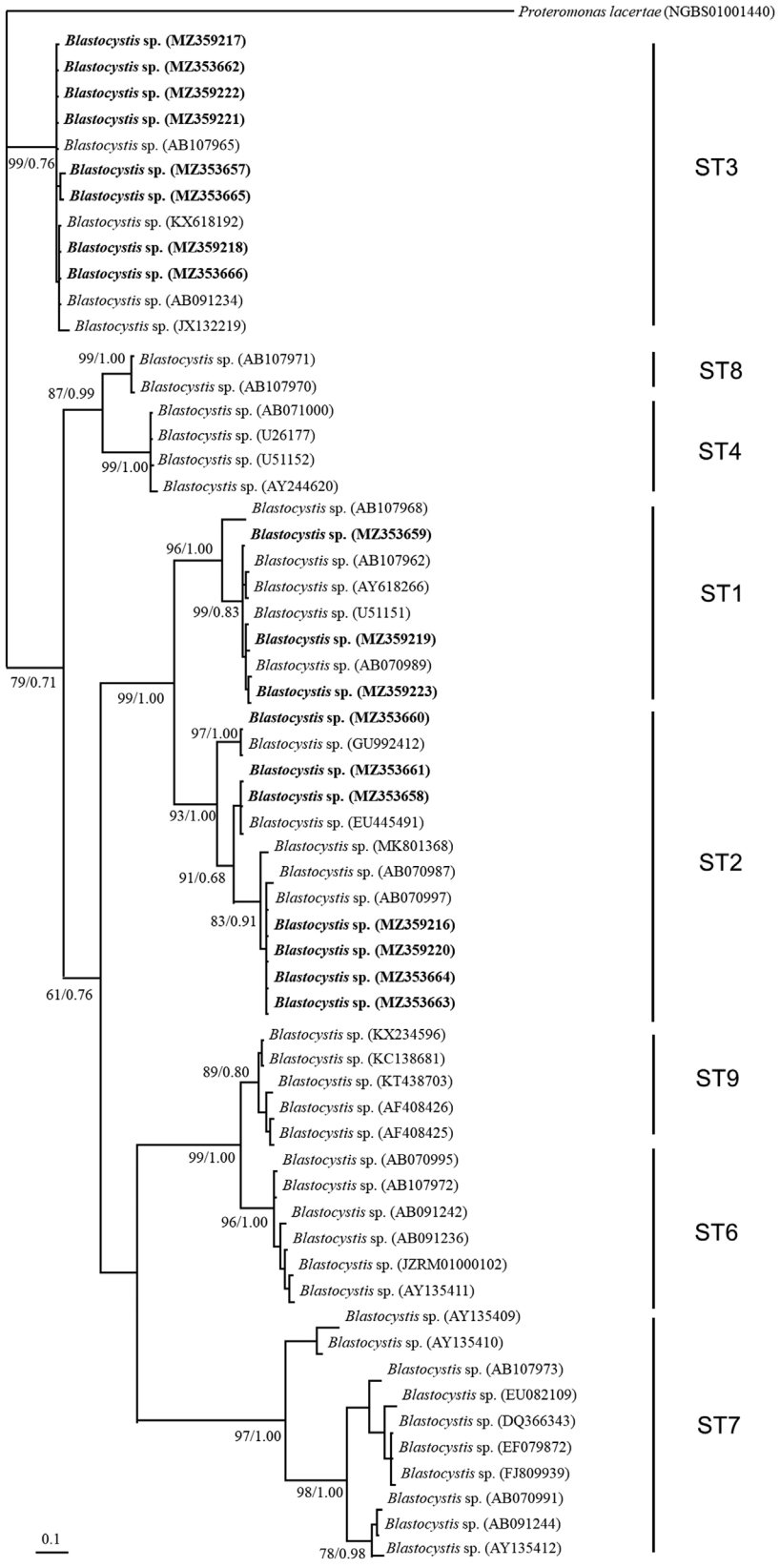

Figure 2 - Phylogenetic inference of *SSUrDNA* gene partial sequence of *Blastocystis* STs1-4 and STs 6-9. The orthologous region of *Proteromonas lacertae* was used as the outgroup. The values of the nodes indicate the bootstrap proportions and Bayesian posterior probabilities in the following order: maximum likelihood/Bayesian analysis. The sequences obtained in the present study are indicated in bold.

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ST1_mRNA_Alfuc          --------RPEYNDYYKKTLEVLTTRYGPIYELWWDGANAQP--HMTHVYDWKGWYAILK 198
ST1_50177               --------RPEYNDYYKKTLEVLTTRYGPIYELWWDGANAQP--HMTHVYDWKGWYAILK 198
ST1_50610               --------RPEYNDYYKKTLEVLTTRYGPIYELWWDGANAQP--HMTHVYDWKGWYAILK 198
ST1_JOEL36              --------RPEYNDYYKKTLEVLTTRYGPIYELWWDGANAQP--HMTHVYDWKGWYAILK 198
ST2_Joel40              --------RPEYNDYYKKTLDVLTSRYGPIYELWWDGANAKE--HMTHVYDWKGWYEILK 197
ST2_Flemming_NCBI       --------RPEYNDYYKKTLDVLTSRYGPIYELWWDGANAKE--HMTHVYDWKGWYEILK 197
ST2_Joel29B             --------RPEYNDYYKKTLDVLTSRYGPIYELWWDGANAKE--HMTHVYDWKGWYAILK 197
ST2_CMX11               --------RPEYNDYYKKTLDVLTSRYGPIYELWWDGANAKE--HMTHVYDWKGWYEILK 197
ST2_Joel19B             --------RPEYNDYYKKTLDVLTSRYGPIYELWWDGANAKE--HMTHVYDWKGWYEILK 197
ST2_Joel5B              --------RPEYNDYYKKTLDVLTSRYGPIYELWWDGANAKE--HMTHVYDWKGWYAILK 197
ST3_Joel42              --------RPEYNEYYSHTLEELTTRYGPIYELWWDGANAQQ--HMTHVYDWQGWYKIIK 198
ST3_NCBI                --------RPEYNEYYSHTLEELTTRYGPIYELWWDGANTQQ--HMTHVYDWQGWYKIIK 198
ST3_CMX5                --------RPEYNEYYSHTLEELTTRYGPIYELWWDGANAQQ--HMTHVYDWQGWYKIIK 198
ST3_Joel17A             --------RPEYNEYYSHTLEELTTRYGPIYELWWDGANAQQ--HMTHVYDWQGWYKIIK 198
ST3_Joel43              --------RPEYNEYYSHTLEELTTRYGPIYELWWDGANAQQ--HMTHVYDWQGWYKIIK 198
TR|Q8A085|Q8A085_BACTN  --------SPRYNKFFIRQLTELLTNYGEVHEVWFDGANGEGPNGKKQVYDWDTVYETIH 228
TR|C5NS94|C5NS94_BIFBI  KLPTFKYKATDYGAYMLNQLYELLTEYGDISEVWFDGAQGNT--AGTEHYDYGVFYEMIR 725
TR|Q9Z4I9|Q9Z4I9_STRSQ  KLPTFTVMADDYDAYYLNQLYEIFTQYGPIEELWLDGANPWSGSGITQKYNVKQWFDMVK 327
                                  *. :  . *  : :.** : *:* ***:       .. *:    :  ::

ST1_mRNA_Alfuc          KNQPQCLGGGCGGDN-DSFDCGPDTAWGKTESGLGREENWNFHAPSVE------------ 245
ST1_50177               KNQPQCLGGGCGGDN-DSFDCGPDTAWGKTESGLGREENWNFHAPSVE------------ 245
ST1_50610               KNQPQCLGGGCGGDN-DSFDCGPDTAWGKTESGLGREENWNFHAPSVE------------ 245
ST1_JOEL36              KNQPQCLGGGCGGDN-DSFDCGPDTAWGKTESGLGREENWNFHAPSVE------------ 245
ST2_Joel40              KNQPQCLGGGCGGDN-DSFDCGPDTAWGKTESGLGKEENWNFHAPSVE------------ 244
ST2_Flemming_NCBI       KNQPQCLGGGCGGDN-DSFDCGPDTAWGKTESGLGKEENWNFHAPSVE------------ 244
ST2_Joel29B             KNQPQCLGGGCGGDN-DSFDCGPDTAWGKTESGLGKEENWNFHAPSVE------------ 244
ST2_CMX11               KNQPQCLGGGCGGDN-DSFDCGPDTAWGKTESGLGKEENWNFHAPSVE------------ 244
ST2_Joel19B             KNQPQCLGGGCGGDN-DSFDCGPDTAWGKTESGLGKEENWNFHAPSVE------------ 244
ST2_Joel5B              KNQPQCLGGGCGGDN-DSFDCGPDTAWGKTESGLGKEENWNFHAPSVE------------ 244
ST3_Joel42              RNQPQCLGGGCGGDEAGIFDCGPDTAWGQTESGLGKEENWNFHTPSVE------------ 246
ST3_NCBI                RNQPQCLGGGCGGDEAGIFDCGPDTAWGQTESGLGKEENWNFHTPSVE------------ 246
ST3_CMX5                RNQPQCLGGGCGGDEAGIFDCGPDTAWGQTESGLGKEENWNFHTPSVE------------ 246
ST3_Joel17A             RNQPQCLGGGCGGDEAGIFDCGPDTAWGQTESGLGKEENWNFHTPSVE------------ 246
ST3_Joel43              RNQPQCLGGGCGGDEAGIFDCGPDTAWGQTESGLGKEENWNFHTPSVE------------ 246
TR|Q8A085|Q8A085_BACTN  RLQPKAVMAI----------MGDDIRWVGNESGLGRETEWSTTVLTPEIYARADKNNKKL 278
TR|C5NS94|C5NS94_BIFBI  RLQPQAIQAN----------AAYDARWVGNEDGWARQTEWSPQAAYNDGV---DKVSLKP 772
TR|Q9Z4I9|Q9Z4I9_STRSQ  ALSPNTVV----------FQGPQGVRWVGNEGGTARETEWSVTPHATDPWTGLGS----L 373
                          .*: :               .  *  .*.* .:: :*.      :
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Figure 3 - Sequence alignment by ClustalW. Only the region corresponding to the catalytic sites of the α-L-fucosidases of the GH29 family is shown. To compare the region of catalytic sites, our sequences were aligned with three α-L-fucosidases of the GH29 family from the UniProt database: *Bacteroides thetaiotaomicron* Q8A085, *Bifidobacterium bifidum* C5NS94, and *Streptomyces sp* Q9Z4I9. The catalytic site is shown with the nucleophilic asp residue (D) in red and the general acid/base Glu residue (E) in blue.

our ALFucs were compared with α-L-fucosidases of the GH29 family of homologous bacteria deposited in the databases. In the present study, we identified the conserved nucleophilic residue D and the acid/base residue E in our sequences. Our results are consistent with the catalytic mechanism described in bacteria for α-L-fucosidase GH29, which requires two amino acid residues, one of which plays the overall acid/base role, while the other acts as a nucleophile[32].

In our study, we could only identify a similar galactose-binding domain in ST1 and ST2. In *Akkermansia muciniphila,* proteins with a similar galactose-binding domain involved in mucin degradation play a significant role in host intestinal health, as they downregulate hydrolytic activity in regions with injured tissues, improving tissue regeneration and wound healing[33]. In *Ruminococcus gnavus*, another human gut symbiont commonly associated with inflammatory bowel disease, the ability to grow with mucin as the sole carbon source depends on the strain and is related to gene groups, including ALFuc proteins with galactose-like binding domain[34]. In addition, it has been observed that the absence of beta domains of analogous galactose-like binding with a topology like a gelatin roll is related to lower hydrolytic activity of β-galactosidase in *Bacillus circulans*[35]. It has been suggested that this domain helps galactosyl-lactose molecules to be correctly targeted within the active site to efficiently hydrolyze thus producing galactose/glucose and inhibiting the accumulation of galacto-oligosaccharides (GOS)[35,36]. In the present study, the ALFuc of *Blastocystis* ST3 showed a negative result for a similar domain of galactose binding, possibly related to affinity/activity for the carbohydrate as mentioned in the examples above. But these data will have to be analyzed with the characterized protein.

Blastocystis ST have been described "as divergent as species or even genera"[4] capable of colonizing a wide variety of hosts[1]. Therefore, it is not surprising that *Blastocystis* is considered the most widespread intestinal micro-eukaryote, with an almost complete lack of host specificity. Due to its potential impact on human health, significant efforts have been made to associate the genetic diversity of *Blastocystis* with gastrointestinal clinical manifestations of zoonotic transmission by analyzing

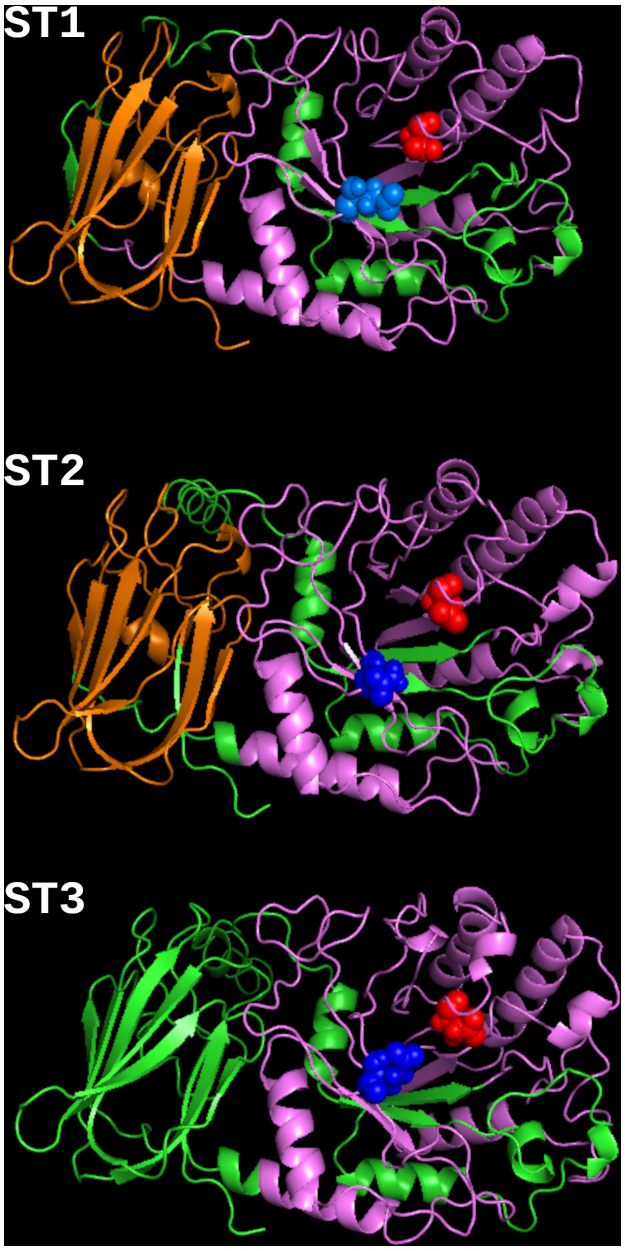

Figure 4 - 3D structures of the ALFuc of *Blastocystis* STs1-3. The image shows the ALFuc domain, formed by five α-helix structures (magenta color), the C-type domain F5/8 or similar galactose-binding domain, formed by five β-strand structures (orange color), in ST1 and ST2. In addition, the catalytic site of the GH29 family, consisting of the nucleophilic residue D (red spheres) and the acid/base residue E (blue spheres), is observed. For ST3, the Pfam result for the similar galactose-binding domain was negative.

different molecular markers between geographically distant populations or isolated from symptomatic and asymptomatic patients[37-39]. Although these studies have demonstrated surprising findings, it was not possible to identify a clear association with pathogenic signatures. A remarkable finding in our study is the conserved topology in the phylogenetic tree of the *alfuc* gene, with the topology described by the subunits of the *nad* gene for the same STs, and the grouping for STs1–4 and STs6–9, with the classical clustering obtained with the *SSUr-RNA* genes[28].

In this study, a genomic region containing the *alfuc* gene was amplified using four pairs of degenerate primers designed to obtain the entire gene for comparative functional analysis rather than to identify a phylogenetic marker. The main limitations and advantages of this strategy should be pointed out to avoid possible biases or to be addressed by future studies.

The primers were designed to amplify regions of interest only for ST1–3. As most of the samples analyzed in global studies are of human origin and "more than 90% of human strains belong to ST1–4"[4], these primers may be advantageous if the study entails ST1–3 or a phylogenetically close ST. However, problems can arise when analyzing the most divergent STs, especially those that colonize amphibians, reptiles, or insects. Unlike previous studies that used DNA extracted from stool samples to evaluate new phylogenetic markers, we used DNA extracted from xenic cultures. The first approach represents the most challenging limitation for field studies using single-copy markers, as they are more susceptible to biases associated with a low parasite load in the sample, e.g., a recent report that analyzed DNA from human stool samples ruled out eight of the 12 new markers evaluated for *Blastocystis* because it was not possible to obtain quality PCR amplification products[2]. Although xenic isolation increases the number of parasites and, consequently, the number of positive samples, this procedure favors the selection of specific subtypes[13], a particular problem for studies designed to describe genetic variability in a particular population. To solve this problem, new studies could implement the next-generation sequencing approaches used to study the genetic diversity of *Blastocystis* subtypes in humans and animals[40] and target new genes such as *alfuc* or implement new available technologies to obtain complete genetic sequences[4], as has already been implemented for SSU-rRNA genes.

CONCLUSION

Our experimental results showed, for the first time that ALFuc is a secretion product of *Blastocystis* sp., which could have a relevant role during intestinal colonization; however, further studies are required to clarify this condition. Furthermore, the *alfuc* gene is a promising candidate to act a phylogenetic marker according to the resulting subtypes, and needs to be investigated to clarify whether the same behavior occurs with other subtypes.

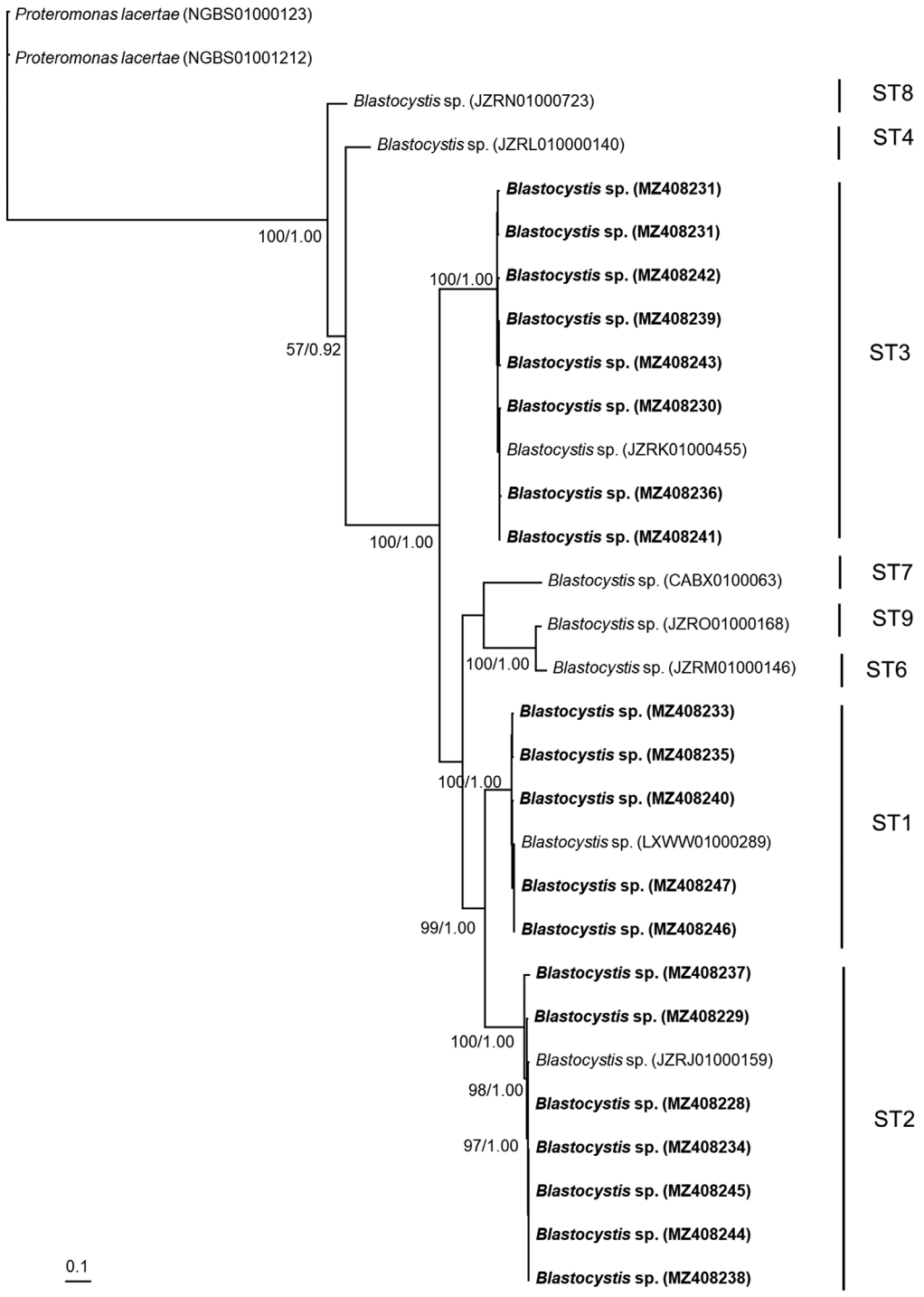

Figure 5 - Phylogenetic inference of the complete *alfuc* genes from *Blastocystis* STs1–4 and STs 6–9. The orthologous region of *Proteromonas lacertae* was used as the outgroup. The values of the nodes indicate the bootstrap proportions and Bayesian posterior probabilities in the following order: maximum likelihood/Bayesian analysis. The sequences obtained in the present study are indicated in bold.

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Salvador Zubirán, Mexico City, for his support in the analysis of the predicted ALFuc sequences. Os nomes dos locais estão em itálico e deveriam ter sido deixados em espanhol

AUTHORS' CONTRIBUTIONS

JMO, WAMF, and PM conceptualized the study. JMO, WAMF, and MRV performed the experimental work and collected the data. JMO, WAMF, and FMH analyzed the data. JMO and WAMF performed the bioinformatics analyses. JMO and WAMF drafted the manuscript, which was reviewed by PM, AOD, AF, and GAO. PM supervised the project, experimental design, data collection and analysis, and manuscript preparation. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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REFERENCES

1. Tan KS. New insights on classification, identification, and clinical relevance of Blastocystis spp. Clin Microbiol Rev. 2008;21:639-65.
2. Stensvold CR, Clark CG. Pre-empting Pandora's box: blastocystis subtypes revisited. Trends Parasitol. 2020;36:229-32.
3. Maloney JG, Santin M. Mind the gap: new full-length sequences of blastocystis subtypes generated via Oxford Nanopore Minion Sequencing allow for comparisons between full-length and partial sequences of the small subunit of the ribosomal RNA gene. Microorganisms. 2021;9:997.
4. Stensvold CR, Tan KS, Clark CG. Blastocystis. Trends Parasitol. 2020;36:315-6.
5. Maloney JG, Molokin A, Cunha MJ, Cury MC, Santin M. Blastocystis subtype distribution in domestic and captive wild bird species from Brazil using next generation amplicon sequencing. Parasite Epidemiol Control. 2020;9:e00138.
6. Yoshikawa H, Koyama Y, Tsuchiya E, Takami K. Blastocystis phylogeny among various isolates from humans to insects. Parasitol Int. 2016;65:750-9.
7. Martínez-Ocaña J, Maravilla P, Olivo-Díaz A. Interaction between human mucins and parasite glycoproteins: the role of lectins and glycosidases in colonization by intestinal protozoa. Rev Inst Med Trop Sao Paulo. 2020;62:e64.
8. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 2014;42:D490-5.
9. Sakurama H, Tsutsumi E, Ashida H, Katayama T, Yamamoto K, Kumagai H. Differences in the substrate specificities and active-site structures of two α-L-fucosidases (glycoside hydrolase family 29) from Bacteroides thetaiotaomicron. Biosci Biotechnol Biochem. 2012;76:1022-4.
10. Liu TW, Ho CW, Huang HH, Chang SM, Popat SD, Wang YT, et al. Role for alpha-L-fucosidase in the control of Helicobacter pylori-infected gastric cancer cells. Proc Natl Acad Sci U S A. 2009;106:14581-6.
11. Luijkx YM, Bleumink NM, Jiang J, Overkleeft HS, Wösten MM, Strijbis K, et al. Bacteroides fragilis fucosidases facilitate growth and invasion of Campylobacter jejuni in the presence of mucins. Cell Microbiol. 2020;22:e13252.
12. Curiel JA, Peirotén A, Landete JM, Ruiz de la Bastida A, Langa S, Arqués JL. Architecture insight of Bifidobacterial α-L-Fucosidases. Int J Mol Sci. 2021;22:8462.
13. Clark CG, Stensvold CR. Blastocystis: isolation, xenic cultivation, and cryopreservation. Curr Protoc Microbiol. 2016;43:20A.1.1-8.
14. Wawrzyniak I, Texier C, Poirier P, Viscogliosi E, Tan KS, Delbac F, et al. Characterization of two cysteine proteases secreted by Blastocystis ST7, a human intestinal parasite. Parasitol Int. 2012;61:437-42.
15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.
16. UniProtKB: D8M3D3 (D8M3D3_BLAHO). [cited 2022 Apr 25]. Available from: https://www.uniprot.org/uniprot/D8M3D3
17. Swiss Institute of Bioinformatics. Expasy: Compute pI/Mw tool. [cited 2022 Apr 25]. Available from: https://web.expasy.org/compute_pi/
18. Matsumoto H, Haniu H, Komori N. Determination of protein molecular weights on SDS-PAGE. Methods Mol Biol. 2019;1855:101-5.
19. Tsang VC, Peralta JM, Simons AR. Enzyme-linked immunoelectrotransfer blot techniques (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. Methods Enzymol. 1983;92:377-91.
20. Santín M, Gómez-Muñoz MT, Solano-Aguilar G, Fayer R. Development of a new PCR protocol to detect and subtype Blastocystis spp. from humans and animals. Parasitol Res. 2011;109:205-12.
21. National Center for Biotechnology Information. Blastocystis sp. ATCC 50177/Nand II strain NandII AV274scaffold_40, whole

genome shotgun sequence. [cited 2022 Apr 25]. Available from: https://www.ncbi.nlm.nih.gov/nuccore/LXWW01000289.1

22. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. The I-TASSER Suite: protein structure and function prediction. Nat Methods. 2015;12:7-8.
23. Schrödinger. PyMOL. [cited 2022 Apr 25]. Available from: https://pymol.org/2/
24. National Center for Biotechnology Information. Protein. [cited 2022 Apr 25]. Available from: https://www.ncbi.nlm.nih.gov/protein/
25. European Molecular Biology Laboratory. Pfam. [cited 2022 Apr 25]. Available from: http://pfam.xfam.org/
26. Ezawa I, Sawai Y, Kawase T, Okabe A, Tsutsumi S, Ichikawa H, et al. Novel p53 target gene FUCA1 encodes a fucosidase and regulates growth and survival of cancer cells. Cancer Sci. 2016;107:734-45.
27. Technical University of Denmark. DTU health tech: SignalP - 5.0. [cited 2022 Apr 25]. Available from: https://services.healthtech.dtu.dk/service.php?SignalP-5.0
28. Alfellani MA, Taner-Mulla D, Jacob AS, Imeede CA, Yoshikawa H, Stensvold CR, et al. Genetic diversity of Blastocystis in livestock and zoo animals. Protist. 2013;164:497-509.
29. Jacob AS, Andersen LO, Bitar PP, Richards VP, Shah S, Stanhope MJ, et al. Blastocystis mitochondrial genomes appear to show multiple independent gains and losses of start and stop codons. Genome Biol Evol. 2016;8:3340-50.
30. Gentekaki E, Curtis BA, Stairs CW, Klimeš V, Eliáš M, Salas-Leiva DE, et al. Extreme genome diversity in the hyper-prevalent parasitic eukaryote Blastocystis. PLoS Biol. 2017;15:e2003769.
31. Eme L, Gentekaki E, Curtis B, Archibald JM, Roger AJ. Lateral gene transfer in the adaptation of the anaerobic parasite Blastocystis to the gut. Curr Biol. 2017;27:807-20.
32. Tarling CA, He S, Sulzenbacher G, Bignon C, Bourne Y, Henrissat B, et al. Identification of the catalytic nucleophile of the family 29 alpha-L-fucosidase from Thermotoga maritima through trapping of a covalent glycosyl-enzyme intermediate and mutagenesis. J Biol Chem. 2003;278:47394-9.
33. Kosciow K, Deppenmeier U. Characterization of a phospholipid-regulated β-galactosidase from Akkermansia muciniphila involved in mucin degradation. Microbiologyopen. 2019;8:e00796.
34. Crost EH, Tailford LE, Le Gall G, Fons M, Henrissat B, Juge N. Utilisation of mucin glycans by the human gut symbiont Ruminococcus gnavus is strain-dependent. PLoS One. 2013;8:e76341.
35. Song J, Imanaka H, Imamura K, Minoda M, Yamaguchi S, Nakanishi K. The discoidin domain of Bacillus circulans β-galactosidase plays an essential role in repressing galactooligosaccharide production. Biosci Biotechnol Biochem. 2013;77:73-9.
36. Yin H, Pijning T, Meng X, Dijkhuizen L, van Leeuwen SS. Biochemical characterization of the functional roles of residues in the active site of the β-Galactosidase from Bacillus circulans ATCC 31382. Biochemistry. 2017;56:3109-18.
37. Villalobos G, Orozco-Mosqueda GE, Lopez-Perez M, Lopez-Escamilla E, Córdoba-Aguilar A, Rangel-Gamboa L, et al. Suitability of internal transcribed spacers (ITS) as markers for the population genetic structure of Blastocystis spp. Parasit Vectors. 2014;7:461.
38. Alarcon-Valdes P, Villalobos G, Martinez-Flores WA, Lopez-Escamilla E, Gonzalez-Arenas NR, Romero-Valdovinos M, et al. Can the pyruvate: ferredoxin oxidoreductase (PFOR) gene be used as an additional marker to discriminate among Blastocystis strains or subtypes? Parasit Vectors. 2018;11:564.
39. Higuera A, Muñoz M, López MC, Reyes P, Urbano P, Villalobos O, et al. Succinate dehydrogenase gene as a marker for studying Blastocystis genetic diversity. Heliyon. 2020;6:e05387.
40. Maloney JG, Cunha MJ, Molokin A, Cury MC, Santin M. Next-generation sequencing reveals wide genetic diversity of Blastocystis subtypes in chickens including potentially zoonotic subtypes. Parasitol Res. 2021;120:2219-31.

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