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Glycoside hydrolase family 13 α-glucosidases encoded by *Bifidobacterium breve* UCC2003; A comparative analysis of function, structure and phylogeny

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ABSTRACT

Bifidobacterium breve is a noted inhabitant and one of the first colonizers of the human gastro intestinal tract (GIT). The ability of this bacterium to persist in the GIT is reflected by the abundance of carbohydrate-active enzymes that are encoded by its genome. One such family of enzymes is represented by the α-glucosidases, of which three, Agl1, Agl2 and MelD, have previously been identified and characterized in the prototype *B. breve* strain UCC2003. In this report, we describe an additional *B. breve* UCC2003-encoded α-glucosidase, along with a *B. breve* UCC2003-encoded α-glucosidase-like protein, designated here as Agl3 and Agl4, respectively, which together with the three previously described enzymes belong to glycoside hydrolase (GH) family 13.
Agl3 was shown to exhibit hydrolytic specificity towards the α-(1 → 6) linkage present in palatinose; the α-(1 → 3) linkage present in turanose; the α-(1 → 4) linkages found in maltotriose and maltose; and to a lesser degree, the α-(1 → 2) linkage found in sucrose and kojibiose; and the α-(1 → 5) linkage found in leucrose. Surprisingly, based on the substrates analyzed, Agl4 did not exhibit biologically relevant α-glucosidic activity.
With the presence of four functionally active GH13 α-glucosidases, *B. breve* UCC2003 is capable of hydrolyzing all α-glucosidic linkages that can be expected in glycan substrates in the lower GIT. This abundance of α-glucosidases provides *B. breve* UCC2003 with an adaptive ability and metabolic versatility befitting the transient nature of growth substrates in the GIT.

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1. Introduction

The human gastrointestinal tract (GIT) is home to more than 100 trillion microorganisms, collectively called the gut microbiota, the majority of which are bacteria that inhabit the colon (Qin et al., 2010). One group of gut commensals is represented by bifidobacteria, being among the first colonizers of the infant gut (Turroni et al., 2012) and commonly present in the distal gut of adult human hosts (Gill et al., 2006; Turroni et al., 2009; Verberkmoes et al., 2009). Bifidobacteria have been associated with various health benefits such as pathogen inhibition/exclusion (Trejo et al., 2006); modulation of the host immune response (Fanning et al., 2012); mitigation of lactose intolerance and the ability to lower serum cholesterol levels in humans (Ooi and Liong, 2010; Russell et al., 2011; Zanotti et al., 2015); along with the prophylaxis and tumor growth regulation of certain cancers (Sivan et al., 2015; Uccello et al., 2012). Bifidobacteria represent Gram-positive, anaerobic, saccharolytic, non-motile, non-sporulating rods that belong to the *Actinobacteria* phylum (Ventura et al., 2004).

The abundance and activity of beneficial elements of the gut microbiota, such as bifidobacteria, can be increased by the use of prebiotics, defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Roberfroid, 2007). Mono- and disaccharide substrates are typically absent from the distal gut, as they are consumed in the small intestine by other gastrointestinal microbes and the host (Schell et al., 2002). Established and emerging saccharidic prebiotics, such as inulin and galacto-oligosaccharides, are resistant to host-encoded digestive enzymes and are thus likely to end up in the distal gut, where they serve as growth substrates for specific beneficial elements of the microbiota, in particular lactobacilli and bifidobacteria (Charalampopoulos and Rastall, 2012). The metabolic capability of bifidobacteria to utilize non-digestible, complex carbohydrates has been well described (Pokusaeva et al., 2011). On average, over 12% of the genes contained by bifidobacterial genomes are predicted to be involved in carbohydrate uptake and metabolism (Milani et al., 2014), being consistent with the

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Table 1
Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant features	Reference or source
Strains		
L. lactis NZ9000	From MG1363, a nisin-inducible overexpression host; *pepN*::*nisRK*	de Ruyter et al. (1996))
L. lactis NZ9000- pNZagl3-His	NZ9000 containing pNZagl3-His	This study
L. lactis NZ9000- pNZagl4-His	NZ9000 containing pNZagl4-His	This study
L. lactis NZ9000- pNZmeld-His	NZ9000 containing pNZmeld-His	O'Connell et al. (2013)
L. lactis NZ9000- pNZagl1	NZ9000 containing pNZagl1-His	Pokusaeva et al. (2009))
E. coli XL1-blue-pQEagl2	XL1-blue containing pQEagl2	Pokusaeva et al. (2009))
B. breve UCC2003	Isolate from a nursling stool	Maze et al. (2007)
Plasmids		
pNZ8150	Cm^r; nisin-inducible translational fusion vector	Mierau and Kleerebezem (2005)
pNZmelD-His	melD with His tag cloned downstream of nisin-inducible promoter on pNZ8150	O'Connell et al. (2013)
pNZagl3-His	agl3 with His tag cloned downstream of nisin-inducible promoter on pNZ8150	This study
pNZagl4-His	agl4 with His tag cloned downstream of nisin-inducible promoter on pNZ8150	This study
pNZagl1	agl1 with His tag cloned downstream of nisin-inducible promoter on pNZ8048	Pokusaeva et al. (2009)
pQEagl2	agl2 with His tag cloned downstream of the isopropyl-ß-D-thiogalactoside (IPTG)-inducible promoter of pQE-70	Pokusaeva et al. (2009)

their ability to grow on various diet- or host-derived carbohydrates (Egan et al., 2014; Milani et al., 2015; McLaughlin et al., 2015; Pokusaeva et al., 2011; Watson et al., 2013). For example, bifidobacteria degrade polysaccharides such as starch and galactan by extracellular glycoside hydrolases (GHs) into oligomeric products (O'Connell Motherway et al., 2008, 2011). Typically, such generated or otherwise present oligo-saccharides will be internalized by carbohydrate-specific ABC-type transporters, and then further hydrolyzed by cytoplasmic GHs, such as α/β-glucosidases and α/β-galactosidases to produce monosaccharides. The *gal*, *mel* and *raf* loci are examples of characterized carbohydrate utilization systems, which specify the abovementioned crucial molecular machinery for the metabolism of galactan, melezitose and raffinose, respectively, by *B. breve* UCC2003 (O'Connell et al., 2013; O'Connell Motherway et al., 2011), a bifidobacterial strain used as a prototype for gut colonization and persistence (Fanning et al., 2012; O'Connell Motherway et al., 2011).

α-Glucosidases (EC number 3.2.1.20) belong to GH families GH4, GH13, GH31, GH63, GH97 or GH122, with a specificity that is primarily directed towards the exohydrolysis of di- and oligo-saccharides. Many α-glucosidases show a preference towards α-(1 → 4) or α-(1 → 6) glucosidic linkages, catalyzing the release of α-glucose from the non-reducing end of their substrates, while retaining the anomeric configuration (Carbohydrate Active Enzymes database http://www.cazy.org/; Larner, 1960; Lombard et al., 2014). Previously, the *B. breve* UCC2003-encoded GH13 α-glucosidases Agl1, Agl2 and MelD had been shown to hydrolyze a range of di- and tri-saccharides with α-glucosidic linkages, thus contributing to this strain's noteworthy growth ability on such substrates (O'Connell et al., 2013; Pokusaeva et al., 2009). In the current study, we describe the identification and characterization of one additional α-glucosidase and an α-glucosidase-like protein encoded by *B. breve* UCC2003.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Bifidobacteria were routinely cultured in either de Man, Rogosa and Sharpe medium (MRS medium; Difco, BD, Le Pont de Claix, France), modified deMan Rogosa Sharpe (mMRS) medium made from first principles (De Man et al., 1960), or reinforced clostridial medium (RCM; Oxoid Ltd., Basingstoke, England), supplemented with 0.05% cysteine-HCl. Bifidobacterial cultures were incubated under anaerobic conditions in a modular, atmosphere-controlled system (Davidson and Hardy, Belfast, Ireland) at 37 °C. *Lactococcus lactis* strains were cultivated in M17 broth containing 0.5% glucose (Terzaghi and Sandine, 1975) at 30 °C aerobically. Where appropriate, growth media contained tetracycline (Tet; 10 μg ml^{-1}), chloramphenicol (Cm; 5 μg ml^{-1} for *L. lactis* and *Escherichia coli*, 2.5 μg ml^{-1} for *B. breve*), erythromycin (Em; 100 μg ml^{-1}) or kanamycin (Kan; 50 μg ml^{-1}) for plasmid selection and maintenance.

2.2. Bioinformatic analysis

All sequences analysed within this study were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) and were submitted and subsequently released in the course of previous studies (Milani et al., 2014; O'Connell Motherway et al., 2011). Database searches were performed aligning our query sequences against the non-redundant database, also accessible at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), using the basic local alignment search tool (BLAST) (Altschul et al., 1990). Sequences were further verified and analyzed using the SeqMan and SeqBuilder programs embedded in the DNAStar suite (version 10.1.2; DNAStar, Madison, WI, USA). The α-glucosidase-encoding genes of *B. breve* UCC2003 of this study were also used to search for homologous genes in the online available type strains of various bifidobacterial species, employing BLASTP searches of translated DNA sequences based on significant hits (e-value < 0.0001) of at least 50% identity across 50% of protein length.

Phylogenetic analysis was conducted using modules included in the MEGA6 analysis suite (Tamura et al., 2011) and consisted in an initial multiple sequence alignment using ClustalW program (Thompson et al., 1994), followed by Neighbor Joining phylogenetic tree construction, using a statistical assessment based on 100 bootstrap replicates.

The template search for the *in silico* structural modeling was conducted using the SWISS-MODEL portal (http://swissmodel.expasy.org/), aligning the sequences of each of the five *B. breve* UCC2003-encoded α-glucosidases against the Protein Data Bank (PDB) database. Resulting matches displaying at least 50% similarity across the full length of the protein (e-value < 0.0001) were chosen as templates. A PDB structure (PDB identifier: 4M56) was selected as the most appropriate template based on sequence similarity with Agl1, Agl2, Agl3, Agl4 and MelD, from Swissmodel template generator (http://swissmodel.expasy.org/). Structural models were built using MODELLERv9.14 (https://salilab.org/modeller/) and the resulting predicted structures were subsequently inspected, refined and aligned using Swiss PDB-viewer software version 4.1 (http://spdbv.vital-it.ch/). The output of the analysis was then visualized using UCSF Chimera software version 1.10.1 (www.cgl.ucsf.edu/chimera/).

2.3. DNA manipulations

Chromosomal DNA was isolated as described previously (Riordan, 1998). Minipreparation of plasmid DNA from *L. lactis* was essentially

carried out using the Roche High Pure Plasmid Isolation Kit (Roche Diagnostics, Basel, Switzerland), with the incorporation of an additional lysis step into the plasmid isolation procedure (Egan et al., 2014). Procedures for DNA manipulations were performed essentially as described previously (Sambrook et al., 1989). Restriction enzymes and T4 DNA ligase were used according to the supplier's instructions (Roche Diagnostics, East Sussex, United Kingdom). The synthetic single-stranded oligonucleotide primers used in this study are listed in supplemental Table S1 and were synthesized by Eurofins (Ebersberg, Germany). Standard PCRs were performed using *Pfu* DNA Polymerase PCR master mix (Thermo Scientific) in a Biometra T3000 thermocycler (Biometra, Göttingen, Germany). PCR products were visualized by ethidium bromide (EtBr) staining following agarose gel electrophoresis (1%). PCR fragments were purified using the High Pure PCR Product Purification Kit (Roche). Plasmid DNA was electrotransformed into *L. lactis* according to published protocols (Wells et al., 1993). Integrity of all constructed plasmids was verified by means of Sanger-based DNA sequencing, performed at Eurofins (Ebersberg, Germany).

2.4. Expression and purification of Agl3 and Agl4

DNA fragments encompassing the full coding sequence of the (predicted) α-glucosidase genes *agl3* and *agl4* were generated by PCR amplification using chromosomal DNA of *B. breve* UCC2003 as a template and employing *Pfu* DNA polymerase and the primer combinations Agl3F and Agl3R, and Agl4F and Agl4R, respectively (see supplemental Table S1), incorporating an EcoRV and an XbaI restriction site at the 5′ end of each forward and reverse primer, respectively, to facilitate cloning. In addition, an in-frame His_{10}-encoding sequence was incorporated into each of the forward primers to allow downstream protein purification using the Ni-nitrilotriacetic acid (NTA) affinity system (Qiagen). The *agl3* and *agl4*-encompassing amplicons were first digested with EcoRV and XbaI, and then ligated into the ScaI- and XbaI-digested nisin-inducible translational fusion plasmid pNZ8150 (Mierau and Kleerebezem, 2005). The ligation mixtures were introduced into *L. lactis* NZ9000 by electrotransformation, and transformants were then selected on the basis of chloramphenicol resistance. The plasmid content of a number of Cm^r transformants was screened by restriction analysis, and the sequence integrity of the generated plasmids (designated pNZAgl3-His, which contains the *agl3* gene, and pNZAgl4-His, containing the *agl4* gene) carried by positively identified clones was verified by sequencing. In order to (over)express and purify the various α-glucosidases, 400 ml of M17 broth supplemented with 0.5% glucose was inoculated with a 2% inoculum of a particular *L. lactis* strain, followed by incubation at 30 °C until an OD_{600} of 0.5 was reached. At that point, protein expression was induced by the addition of purified nisin (5 ng ml^{-1}), and incubation was continued at 30 °C for 1.5 h. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8), and then disrupted with glass beads in a mini-bead beater (BioSpec Products, Bartlesville, OK). Cellular debris was removed by centrifugation. The recombinant Agl3 and Agl4 proteins, with incorporated His_{10} sequence ($Agl3_{His}$ and $Agl4_{His}$), were purified from a crude cell extract using a nickel-nitrilotriacetic acid column (Qiagen GmbH) according to the manufacturer's instructions (QIAexpressionist, June 2003). Elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis, as described previously (Laemmli, 1970), on a 12.5% polyacrylamide gel. Following electrophoresis, the gels were fixed and stained with Coomassie brilliant blue to identify fractions containing the purified protein. Color Prestained Protein Standard, Broad Range (11–245 kDa) (New England BioLabs, Hertfordshire, United Kingdom) were used to estimate the molecular weights of the purified proteins. Protein purification yields were calculated as a percentage of total protein present in the purified fraction over total protein present in crude cell extract. Protein concentrations were determined with the standard Bradford method and employing a bovine serum albumin-based standard curve as a reference (Bradford, 1976).

2.5. Biochemical characterization of bifidobacterial α-glucosidases

The α-glucosidase activity of Agl3 and Agl4 (as well as of Agl1, Agl2 and MelD for certain substrates) was analyzed as described previously (Börnke et al., 2001). For qualitative analysis of catalytic activities exhibited by Agl3 and Agl4, these proteins were each incubated with a potential carbohydrate substrate (as listed in Table S2) at a final concentration of 3 mM for trehalose, trehalulose, sucrose, kojibiose, turanose, nigerose, maltulose, maltose, cellobiose, lactose, leucrose, palatinose, isomaltose, gentiobiose, or melibiose; 2 mM for melezitose, maltotriose, raffinose, panose, or isomaltotriose; 1.5 mM for stachyose, glycogen, and maltotetraose; 1 mM for maltohexaose, maltoheptaose, or maltooctaose; or 1 mg ml^{-1} for maltodextrin. Each of the reactions also contained 20 mM morpholinepropanesulfonic acid (MOPS buffer, pH 7.0) and 50 μl of the purified protein (59 nM or 28 nM of Agl3 or Agl4, respectively), in a final volume of 1 ml. The reactions were incubated at 37 °C for 14 h. Following incubation, reactions were terminated by heat treatment at 99 °C for 15 min (as an exception, due to thermal instability of turanose, reactions involving this carbohydrate were ended by incubation at 62 °C for 15 min). Control reactions were set up for each carbohydrate, by replacing the 50 μl of enzyme with 50 μl of deionized Milli-Q water. Following incubation reactions were stored at −20 °C prior to high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis (see below.

Sucrose (final concentration 50 mM) was used as the substrate for the determination of the pH and temperature optima (pH ranging from 2.5 to 9.5, and a temperature range of 4 °C to 60 °C were tested). Reactions were initiated by the addition of 50 μl of purified Agl3 (89 nM) with 20 mM MOPS buffer in a final reaction volume of 1 ml.

Kinetic constants for Agl3 using sucrose, kojibiose, turanose, maltose, leucrose, palatinose and maltotriose, and for MelD using maltose, palatinose, melezitose and maltotriose were determined by measuring the hydrolysis rates at seven different substrate concentrations, ranging from 1 to 150 mM. Enzymatic activity at particular substrate concentrations were plotted as Lineweaver Burk and Eadie-Hofstee plots. The Eadie–Hofstee plots were used to determine the Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) using the Michaelis–Menten equation:

$$V = V_{max}\,[S]/K_m + [S]$$

where V is the reaction velocity (a function of enzyme concentration), [S] is the substrate concentration, K_m is the substrate concentration at half-maximal velocity, and V_{max} is the maximal velocity. The turnover number (k_{cat}) was determined for each enzyme with each of the studied substrates using the following equation:

$$k_{cat} = V_{max}/E$$

where E is the concentration of enzyme present in the reaction.

Reactions were initiated by the addition of 50 μl purified protein (different concentration of purified protein were used, ranging from 45 nM to 89 nM for Agl3, and 56 nM to 94 nM for MelD) in 20 mM MOPS buffer in a final reaction volume of 1 ml, at the optimal pH and temperature determined for each protein. The final concentration of protein in each reaction was accounted for in the kinetic analysis. The reactions involving sucrose, kojibiose, maltose, leucrose, palatinose, melezitose and maltotriose as substrates were discontinued at different time points (up to 3 min, depending on the concentration of substrate used; at lower substrate concentrations the reaction time was extended) by heat treatment at 99 °C for 15 min. The reaction for turanose was terminated at time points (up to 3 min, dependent on the

concentration of substrate used; at lower substrate concentrations the reaction time was extended) by heat treatment at 62 °C for 15 min. All experiments were performed in duplicate, and the amount of glucose released from each disaccharide substrate was measured by the use of the Glucose Hexokinase Assay Kit (Sigma-Alrich Ireland Ltd., Wicklow, Ireland) according to the manufacturer's instructions.

2.6. HPAEC-PAD analysis

For HPAEC-PAD analysis, a Dionex (Sunnyvale, CA) ICS-3000 system was used. Carbohydrate fractions (25 μl aliquots) were separated on a CarboPac PA1 analytical-exchange column (dimensions, 250 mm by 4 mm) with a CarboPac PA1 guard column (dimensions, 50 mm by 4 mm) and a pulsed electrochemical detector (ED40) in the PAD mode (all from Dionex). Elution was performed at a constant flow-rate of 1.0 ml min^{-1} at 30 °C using the following eluents for the analysis: eluent A, 200 mM NaOH; eluent B, 100 mM NaOH plus 550 mM Na acetate; eluent C, Milli-Q water. The following linear gradient of sodium acetate was used with 100 mM NaOH: from 0 to 50 min, 0 mM; from 50 to 51 min, 16 mM; from 51 to 56 min, 100 mM; from 56 to 61 min, 0 mM. Chromatographic profiles of standard carbohydrates were used for comparison of the results of their breakdown by, where relevant, Agl1, Agl2, Agl3, Agl4 or MelD. Chromeleon software (version 6.70; Dionex Corporation) was used for the integration and evaluation of the chromatograms obtained. A 3 mM, 2 mM, 1.5 mM or 1 mM solution of each of the carbohydrates to be used as reference standards was prepared by dissolving the particular sugar in deionized Milli-Q water. The standards were then sterilized by membrane filtration using Filtropure S filters (pore size, 0.45 μm; Sarstedt, Nümbrecht, Germany) and stored at 4 °C.

3. Results and discussion

3.1. Identification of the agl3 and agl4 genes on the B. breve UCC2003 genome

This study was aimed at the identification and characterization of additional *B. breve* UCC2003-encoded α-glucosidases involved in the degradation of plant-derived carbohydrates, primarily short oligo- and di-saccharides which would be present intracellularly. Three α-glucosidase-encoding genes in the *B. breve* UCC2003 genome (designed *agl1*, *agl2* and *melD*) have previously been identified and characterized (O'Connell et al., 2013; Pokusaeva et al., 2009), the products of which are all members of the GH family 13 (http://www.cazy.org/). The annotated *B. breve* UCC2003 genome (O'Connell Motherway et al., 2011) was analyzed employing the CAZy database (www.cazy.org/) and the EC (enzyme classification) annotation (http://enzyme.expasy.org/), in order to identify additional α-glucosidase-encoding genes in this genome. This analysis yielded four genes, corresponding to locus tags Bbr_0022, Bbr_1143, Bbr_0111 and Bbr_0117, which are predicted to encode proteins with α-glucosidic activity (EC 3.2.1.20). Of these, the genes corresponding to Bbr_0022 and Bbr_1143 were predicted to specify GH31 α-glucosidases, thus being different from the *B. breve* UCC2003 GH13 family members already characterized (Agl1, Agl2 and MelD), and they were therefore considered to be outside the scope of the current study.

The genes associated with locus tags Bbr_0111 and Bbr_0117, designated here as *agl3* and *agl4*, respectively, specify putative GH family 13 α-glucosidases. The *ag13* gene (locus tag Bbr_0111) is 1818 bp in length, encoding a protein of 605 amino acids (deduced molecular mass ~67.087 kDa), whilst the *agl4* gene (locus tag Bbr_0117) is 1890 bp in length, encoding a protein of 629 amino acids (with a presumed molecular mass of ~69.871 kDa).

Both *agl3* and *agl4* are located in the vicinity of the *apuB* gene (locus tag Bbr_0123; Fig. 1), which encodes an extracellular, bifunctional class II amylopullulanase (EC 3.2.1.41), that belongs to the GH13 family, being responsible for the hydrolysis of α-1,4- and α-1,6-glucosidic linkages in starch and related polysaccharides (O'Connell Motherway et al., 2008). The resulting malto-oligosaccharides (of a chain length ranging between 2 and 6) are presumed to be internalized by an ABC-type uptake system and then further degraded to glucose by one or more intracellular α-glucosidases (Lehner et al., 2006; Schönert et al., 2006; Vihinen and Mäntsälä, 1989). The Agl1, Agl2, Agl3, Agl4 and MelD proteins could thus be candidates for such intracellular α-glucosidase activity. Interestingly, analyzed *B. breve* genomes have been shown to encode five putative GH 13 α-glucosidases. On average, this is more than other analyzed bifidobacterial species, of which the majority encode between one and three putative GH 13 α-glucosidases, with the exceptions of *Bifidobacterium callitrichos*, *Bifidobacterium dentium* and *Bifidobacterium gallinarum*, which all are predicted to encode four GH 13 α-glucosidases, along with *Bifidobacterium saeculare* and *Bifidobacterium psychraerophilum* which encode five and six putative GH 13 α-glucosidases, respectively (http://www.cazy.org/; http://www.ncbi.nlm.nih.gov; Milani et al., 2014; Pokusaeva et al., 2011).

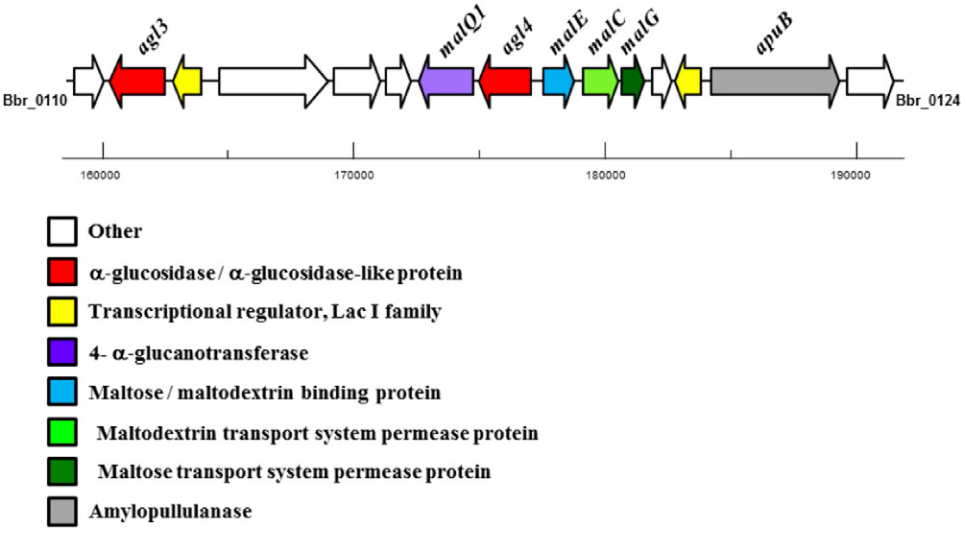


Fig. 1. Visualization of the genetic context of *agl3* and *agl4* on the *B. breve* UCC2003 genome. Each solid arrow represents an open reading frame. The length of the arrows is proportional to the size of the open reading frame and the gene locus name, which is indicative of its putative function, is given at the top.

3.2. Purification, characterization, and substrate specificity of Agl3 and Agl4

In order to verify the predicted glycoside hydrolase function of Agl3 and Agl4, the corresponding genes were cloned into an expression vector to allow (over)expression and purification of the encoded protein (see Materials and Methods). Soluble Agl3 and Agl4 proteins were thus purified, exhibiting molecular masses of approximately 65 kDa and 75 kDa (inclusive of the His_{10}-tag), respectively, when analyzed by SDS-PAGE, consistent with their deduced molecular weights (which are 67.087 kDa and 69.871 kDa, respectively) (Fig. S1). Following purification, on average 25 mg of Agl3 and 13 mg of Agl4 was the yield obtained from 400 ml of induced culture (as described in Materials and Methods).

In order to establish if Agl3 and Agl4 exhibit the expected glycosidic activity, a series of reactions were set up using a wide range of substrates (Tables S2, S3) along with purified Agl3 or Agl4, followed by HPAEC-PAD analysis (as described in Materials and Methods). These analyzes demonstrated that Agl3 is indeed capable of partially or completely hydrolysing several of the tested α-glucosidic containing substrates (Table S3). Surprisingly, when Agl4 was incubated with the substrates available (Table S2) for 14 h, very limited substrate hydrolysis was detected for certain substrates (Table S3; Fig. 2Aii; Bii); the observed activity was nonetheless more than 400-fold lower than that of similar reactions involving Agl3 and was therefore not considered biologically relevant.

Agl3 and Agl4 were shown to be incapable of hydrolysing the β-glucosidic linkages in cellobiose or gentiobiose, or the galactosidic bonds in lactose, melibiose, stachyose or raffinose (Table S3). These results, therefore, confirm our assumption that Agl3 is an enzyme that exclusively exhibits α-glucosidic activity, while Agl4 is a protein (termed here as an α-glucosidase-like protein), which displays a very low level of hydrolytic activity towards exclusively α-glucosidic linkages. It was previously established that the preferred substrates for Agl1 and Agl2 are carbohydrates that contain either an α-(1 → 6) glucosidic linkage as found in palatinose and isomaltose, or an α-(1 → 1) glucosidic linkage found in trehalose and trehalulose (Pokusaeva et al., 2009), while MelD was previously established to be an α-glucosidase with hydrolytic activity towards the α-(1 → 2) and the α-(1 → 3) glucosidic bonds present in sucrose and turanose, respectively (O'Connell et al., 2013).

The relative ability of Agl3, Agl4 and the three previously characterized α-glucosidases from *B. breve* UCC2003 to break down malto-oligosacccharides was tested. The malto-oligosacccharides which were available were maltotriose, maltotetraose, maltohexaose, maltoheptaose and maltooctaose. It had previously been shown that Agl1 and Agl2 are unable to cleave α-(1 → 4) glucosidic linkages, and in accordance to this previous observation these two enzymes were indeed unable to hydrolyse any of the tested malto-oligosaccharides (Table S3). Both Agl3 and MelD were capable of cleaving α-(1 → 4) glucosidic linkages found in maltotriose and maltotetraose, but not of maltohexaose, maltoheptaose and maltooctaose (Table S3). This would imply that the catalytic site of the enzyme is unable to accommodate malto-oligosaccharides with a degree of polymerization greater than four or five. Agl4 was unable to degrade any of the malto-oligosacccharides under the conditions tested (Table S3).

3.3. Determination of the kinetic parameters of Agl3 and MelD

As mentioned above, Agl3 was found to be capable of cleaving a range of substrates. One of the substrates which Agl3 was active against, sucrose, was employed to determine that the pH and temperature optima for this enzyme are pH 6.5 and 37 °C, respectively (Fig. 3). Due to the impaired activity of Agl4 we were unable to establish the pH and temperature optima for this protein, nor any kinetic information. The pH and temperature optima for the previously characterized, recombinantly produced α-glucosidases from *B. breve* UCC2003 (*i.e.* Agl1, Agl2 and MelD) along with the recombinantly produced Agl3, are therefore all within the same range (pH 5.5–7.5 and 30 °C–37 °C) (Fig. 3C). It is assumed that these pH and temperature ranges reflect the cytoplasmic

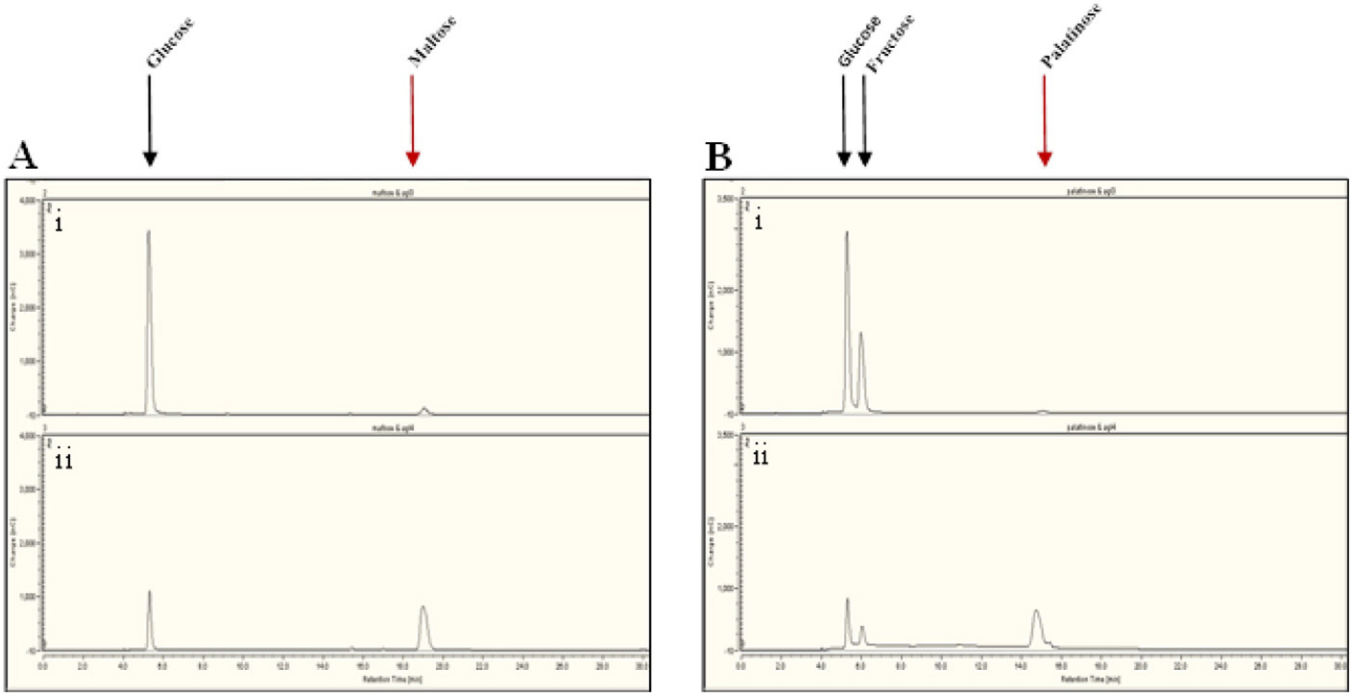
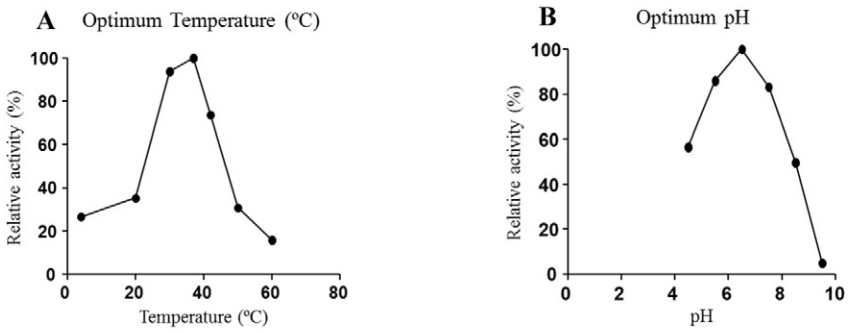


Fig. 2. HPAEC-PAD analysis indicating the breakdown of maltose and palatinose (initial concentration, 1 mg ml^{-1}) by the purified recombinant proteins $Agl3_{His}$ and $Agl4_{His}$ in 20 mM MOPS buffer (pH 7.0) over 14 h. The chromatogram shows results for maltose (A) and palatinose (B) incubated with $Agl3_{His}$ (i) and $Agl4_{His}$ (ii). The liberation of glucose and fructose is visible as chromatographic peaks eluted at 5 and 6 min, respectively. Breakdown products are indicated by black arrows. Chromatographic positions of carbohydrate standards are indicated by red arrows above the chromatogram.



C

	Agl1	Agl2	Agl3	Agl4	MelD
Optimum pH	5.5	5.5	6.5	ND	7.5
Optimum Temperature (ºC)	37	30	37	ND	30

Fig. 3. Effects of temperature (A) and pH (B) on the activity of $Agl3_{His}$. The optimum temperature (A) and pH (B) conditions for catalytic activity, using sucrose as a substrate, were determined to be pH 6.5 and 37 °C. Effects of temperature and pH on $Agl1_{His}$, $Agl2_{His}$, $Agl3_{His}$ and $MelD_{His}$ (C). Temperature and pH optima were not determined for $Agl4_{his}$ due to low hydrolytic activity of the protein.

and environmental conditions of *B. breve* UCC2003. It is worth noting that Agl1, Agl2 and Agl3 are not particularly sensitive to a low pH; under the conditions tested Agl1 maintains close to 100% of its activity when the pH was lowered to 2.5, Agl2 maintains 80% of its optimal activity at pH 2.5, while Agl3 maintains 60% of its optimal activity at pH 4.5 (Fig. 3B).

In order to determine the kinetic parameters of the Agl3 enzyme, we biochemically characterized this glycoside hydrolase using the substrates against which it had shown hydrolytic activity (except for trehalulose, maltulose and nigerose for which we could not obtain sufficient substrate amounts to perform such studies). Kinetic studies were performed to determine V_{max} and K_m values, as well as the rate constants (k_{cat}) and catalytic efficiencies (k_{cat}/K_m) for Agl3. Kinetic studies involving maltose, palatinose, maltotriose and melezitose were carried out to complement previously obtained kinetic data for MelD (O'Connell et al., 2013), while for the kinetic analysis of Agl3, sucrose, kojibiose, turanose, maltose, leucrose, palatinose and maltotriose were used as substrates (Table 3).

Agl3, as outlined above, exhibits high hydrolytic activity against all possible α-glucosidic bonds, except for the α-(1 → 1) bond present in trehalose (Table S3). The data obtained indicates that the preferred bonds cleaved by Agl3 are the α-(1 → 6) bond (palatinose), the α-(1 → 3) bond (turanose), the α-(1 → 4) bond (maltotriose and maltose); and, to a lesser degree, the α-(1 → 2) bond (sucrose and kojibiose) and α-(1 → 5) bond (leucrose) (Table 3).

The broad substrate specificity of Agl3 has not been reported for other bifidobacterial α-glucosidases. In fact, though various publications have focused on α-glucosidic activity in bifidobacteria (Chevalier et al., 1990; Rada, 1997; Vlková et al., 2005), not many reports have dealt with substrate specificities of these enzymes. A similar broad substrate specificity has been documented for α-glucosidases from other bacteria like *Rhizobium* sp. (Berthelot and Delmotte, 1999), and an α-glucosidase isolated from *Bacillus* sp. SAM1606, which can hydrolyse α-(1 → 6), α-(1 → 1), α-(1 → 2) and α-(1 → 4) glucosidic linkages (Noguchi et al., 2003). The common substrate specificity for Agl1, Agl2, MelD and Agl3 towards palatinose has been reported for an α-glucosidase from *Enterobacter sakazakii* which was shown to preferentially hydrolyse α-(1 → 6) bonds (and other α-glucosidic bonds, though with a decreased preference) (Lehner et al., 2006). The overall substrate specificity findings for Agl3 are comparable to what was found for the AglB α-glucosidase isolated and characterized from *Bifidobacterium adolescentis* DSM20083, with which Agl3 shares 61% sequence identity. The AglB enzyme was shown to have a high affinity for α-(1 → 6), α-(1 → 4) and, though to a lesser degree, α-(1 → 2) glucosidic linkages (Van den Broek et al., 2003). Agl3 also exhibits 60% identity to a putative α-glucosidase from *B. dentium* Bd1, of which the corresponding gene (BDP_0624) was noted to be upregulated when grown on maltose along with the upregulation of a putative sugar transporter (Ventura et al., 2009).

Using K_m and k_{cat}/K_m as measures of enzyme affinity for a particular substrate, it can be deduced that (i) the α-(1 → 1) glucosidic bond is primarily cleaved by Agl1 and Agl2; (ii) the α-(1 → 2) glucosidic bond are primarily cleaved by MelD and Agl3; and (iii) the α-(1 → 3), α-(1 → 4) and α-(1 → 5) glucosidic bonds are primarily cleaved by Agl3 (Tables 2, 3).

Agl1 and Agl2 have a noted ability to hydrolyse α-(1 → 6) glucosidic bonds found in isomaltose and palatinose, thus theoretically allowing hydrolysis of any residual α-(1 → 6) branch points on oligosaccharides

Table 2
Agl1 and Agl2 kinetic parameters for various substrates.

Substrate	Agl1				Agl2			
	V_{max}	K_m	k_{cat}	k_{cat}/K_m	V_{max}	K_m	k_{cat}	k_{cat}/K_m
Disaccharides								
Trehalose	8.5 ± 1.28	33.6 ± 4.25	7.3 ± 1.8	0.2 ± 0.08	6.45 ± 0.54	31.15 ± 3.45	5.8 ± 0.33	0.2 ± 0.01
Trehalulose	41.2 ± 5.2	30.2 ± 3.7	35.4 ± 7.76	1.2 ± 0.03	45.1 ± 5.1	22.9 ± 3.5	38.7 ± 5.93	1.7 ± 0.24
Palatinose	14.4 ± 2.78	27.2 ± 1.99	12.4 ± 2.38	0.5 ± 0.12	16.2 ± 2.0	7.1 ± 0.07	13.0 ± 4.53	1.8 ± 0.65
Isomaltose	35.9 ± 3.01	8.3 ± 0.02	30.8 ± 2.58	3.7 ± 0.32	42.3 ± 2.7	9.0 ± 0.8	36.3 ± 2.28	4.0 ± 0.09
Oligosaccharides								
Panose	30.8 ± 3.05	10.4 ± 1.21	28.7 ± 3.52	2.8 ± 1.02	33.0 ± 4.42	15.0 ± 3.56	30.0 ± 3.94	2.0 ± 0.07
Isomaltotriose	14.5 ± 0.86	16.7 ± 1.89	12.5 ± 0.74	0.8 ± 0.13	14.0 ± 1.83	5.5 ± 0.99	12.0 ± 1.57	2.5 ± 1.06

[a] V_{max} values are expressed as μmol min^{-1} mg^{-1}, K_m values are expressed as mM, k_{cat} values are expressed as s^{-1}, and k_{cat}/K_m values are expressed as mM^{-1} s^{-1}. All values are means from two experiments ± standard errors.

Table 3
Agl3 and MelD kinetic parameters for various substrates.

Substrate	Agl3				MelD			
	V_{max}	K_m	k_{cat}	k_{cat}/K_m	V_{max}	K_m	k_{cat}	k_{cat}/K_m
Disaccharides								
Sucrose	96.29 ± 1.8	39.04 ± 0.53	106.45 ± 2.0	2.73 ± 0.01	7.75 ± 0.07	19.3 ± 0.28	8.07 ± 0.07	0.42 ± 0.01
Kojibiose	56.82 ± 3.17	44.14 ± 1.58	64.82 ± 3.61	1.47 ± 0.03	N/A	N/A	N/A	N/A
Turanose	27.75 ± 0.15	3.7 ± 0. 1	31.66 ± 0.17	8.56 ± 0. 28	12.66 ± 1.59	91.93 ± 1.07	13.16 ± 1.61	0.14 ± 0.01
Maltose	58.88 ± 1.69	22.47 ± 3.91	67.18 ± 1.93	3.1 ± 0.63	54.03 ± 0.24	46.11 ± 1.78	49.05 ± 1.89	0.91 ± 0.04
Leucrose	36.2 ± 1.9	46.7 ± 3.3	41.3 ± 2.17	0.89 ± 0.02	N/A	N/A	N/A	N/A
Palatinose	14.2 ± 1.0	2.4 ± 0. 1	16.2 ± 1.14	6.78 ± 0. 76	5.54 ± 0.025	43.76 ± 4.4	5.89 ± 0.03	0.14 ± 0.01
Oligosaccharides								
Melezitose	N/A	N/A	N/A	N/A	54.31 ± 0.65	4.05 ± 0.12	57.77 ± 0.69	14.06 ± 0.04
Maltotriose	43.41 ± 1.3	27.24 ± 0.57	49.52 ± 1.48	1.82 ± 0.02	36.99 ± 0.48	3.29 ± 0.02	39.35 ± 0.51	11.98 ± 0.12

[a] V_{max} values are expressed as μmol min^{-1} mg^{-1}, K_m values are expressed as mM, k_{cat} values are expressed as s^{-1}, and k_{cat}/K_m values are expressed as mM^{-1} s^{-1}. All values are means from two experiments ± standard errors.

that have been internalized following their production by the extracellular pullulanase ApuB from the degradation of starch, pullulan, amylopectin or glycogen. All four functional α-glucosidases (Agl1, Agl2, Agl3 and MelD) are effective at hydrolysing the α-(1 → 6) Glc-Fru glucosidic bond found in palatinose. This common preference may be linked to the fact that palatinose is a lot more resistant to acid and invertase hydrolysis in the gut (Lina et al., 2002), and may thus be present in the large intestine in higher quantities than sucrose for example, where it may act as a growth substrate for bifidobacteria.

3.4. Phylogenetic analysis, in silico modeling and amino acid alignment of B. breve UCC2003-encoded α-glucosidases and an α-glucosidase-like protein

Genome sequences of 47 bifidobacterial type strains were inspected using BLAST in order to reveal the presence of putative homologs of GH13 α-glucosidase-encoding genes. Out of the positive hits, 108 were chosen to be included as deduced proteins in the phylogenetic analysis (based on a minimum of 50% identity and 50% coverage with

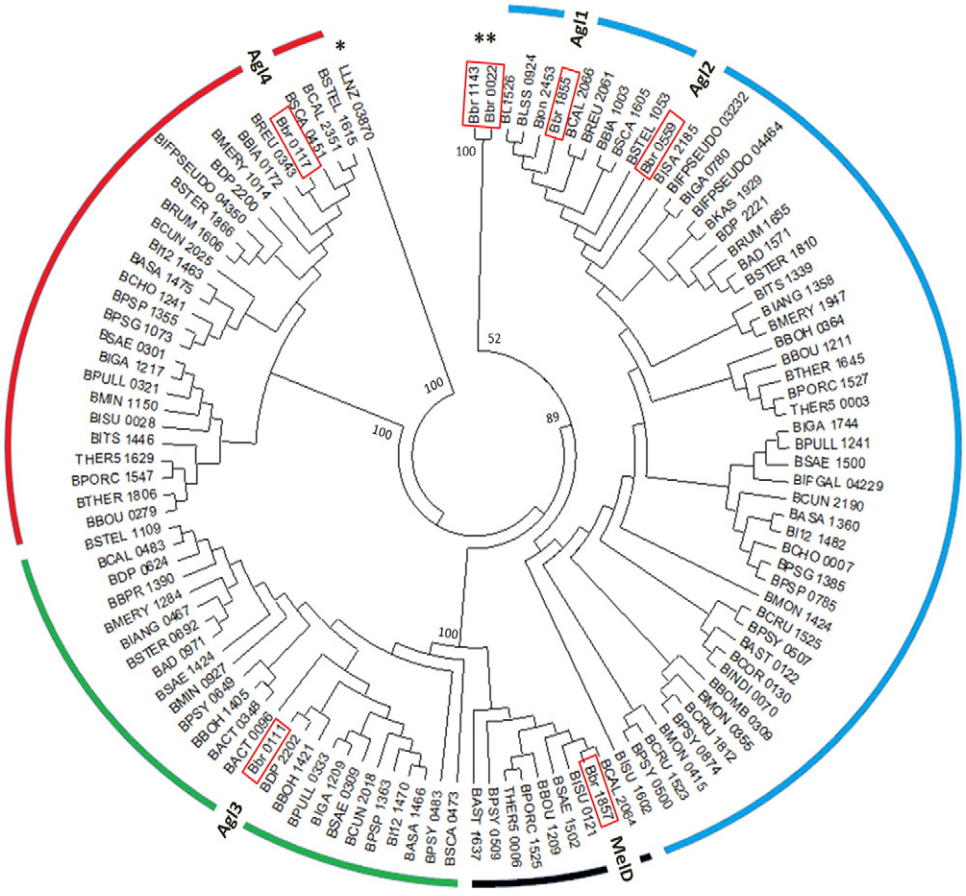


Fig. 4. Rooted neighbor-joining tree based on the alignment of 108 putative α-glucosidase proteins from type *Bifidobacterium spp.* strains, to the four *B. breve* UCC2003-encoded α-glucosidases and the α-glucosidase-like protein. Rooted neighbor-joining tree using a statistical assessment based on 100 bootstrap replicates. All 108 proteins were chosen based on a minimum of 50% of identity and 50% of coverage towards any of the five *B. breve* UCC2003-encoded proteins. Three additional amino acid sequences were added to the phylogenetic analysis; two putative *B. breve* UCC2003-encoded GH31 α-glucosidases, along with a putative *Lactococcus lactis* NZ-encoded GH13 α-glucosidase as an outgroup. * GH 13 α-glucosidase from *Lactococcus lactis* NZ9000 ** GH 31 α-glucosidases from *Bifidobacterium breve* UCC2003.

Agl4 or any one of the four *B. breve* UCC2003-encoded GH13 α-glucosidases). Three additional amino acid sequences were added to the phylogenetic analysis: the two putative *B. breve* UCC2003-encoded GH31 α-glucosidases, along with a putative *L. lactis*-encoded GH13 α-glucosidase as an outgroup.

As can be seen in Fig. 4, Agl1 and Agl2 cluster together on the same branch point, demonstrating their close phylogenetic relationship. Agl3 and MelD are closely related from a phylogenetic perspective, clustering within a sister group of one branch, indicating that they belong to a common lineage. As established experimentally, there is considerable functional similarity between Agl1 and Agl2, and though to a somewhat lesser degree between MelD and Agl3. The Agl4 protein clusters separately from the four *B. breve* UCC2003-encoded α-glucosidases, consistent with its apparently deviating functional properties. Surprisingly, 26 of the bifidobacterial type strains screened were shown to encode a homolog of the α-glucosidase-like protein, clustering on the same branch point as Agl4.

The significance of α-glucosidases to the bifidobacterial species is apparent; all genomes of the type strains screened contain at least one putative α-glucosidase-encoding gene, but more commonly encompass several such genes (Fig. 4).

A comparative structural analysis conducted on the four α-glucosidases (Agl1, Agl2, Agl3 and MelD) and the α-glucosidase-like protein Agl4, was performed using an *in silico* approach, in order to reveal and study possible relationships between protein sequence, structure and functional specificities. Members of the GH13 family possess a typical α-glucosidase structure with a catalytic domain (domain A in Figs. 5 and 6) that is present in the form of a $(\beta/\alpha)_8$-barrel, along with a C-terminal antiparallel β-sheet domain (domain C in Figs. 5 and 6); these structures can be observed in each of the five protein models (MacGregor et al., 2001; Møller et al., 2012; Watanabe et al., 2001). The active site is located at the C-terminal end of the β-barrel strands in domain A, encompassing three conserved catalytic residues (Asp296, Asp462 and Glu374 in the multiple alignment consensus of Fig. 6e) (Hondoh et al., 2008; Møller et al., 2012; Watanabe et al., 2001). Domains B and B′ are known to be involved in substrate interaction (Møller et al., 2012), and a relatively low degree of conservation of these particular domains exists across the four *B. breve* UCC2003-

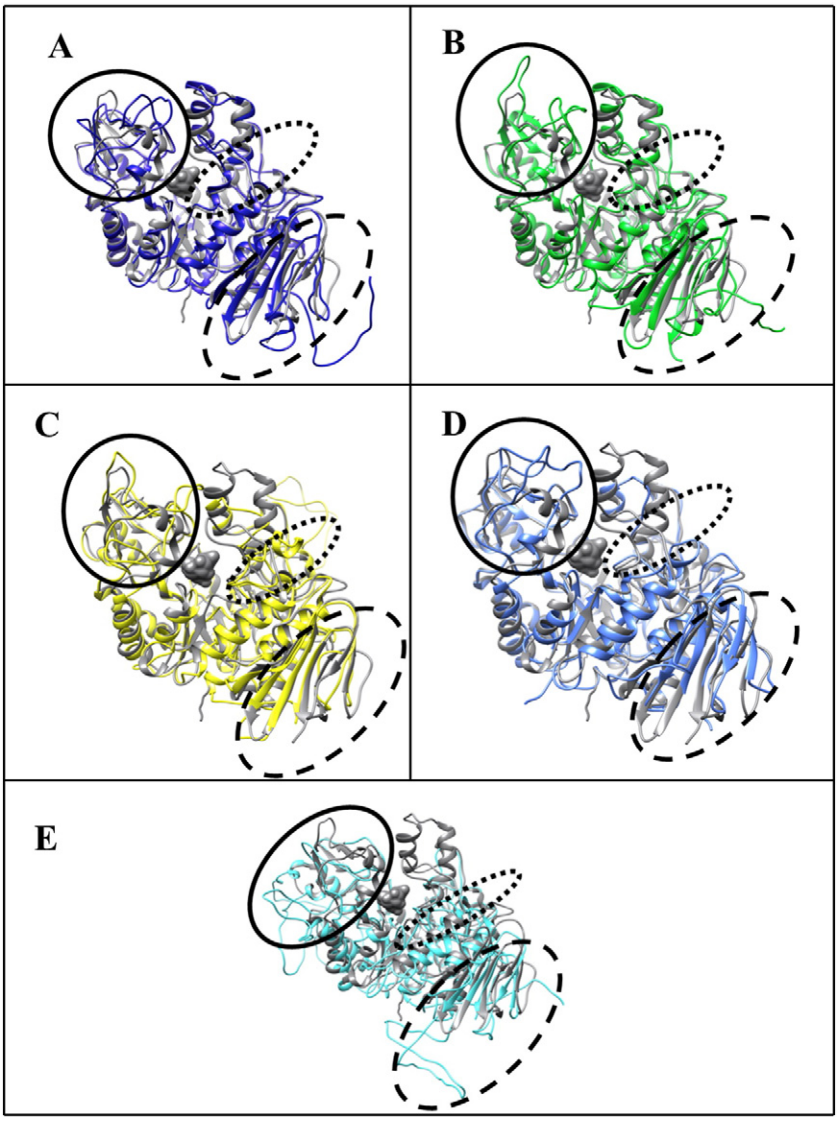


Fig. 5. Predicted tertiary protein structure of the five putative *B. breve* UCC2003-encoded GH31 α-glucosidases: A.) Agl1, B.) Agl2, C.) Agl3, D.) MelD and E.) Agl4. The sequence model of the Protein Data Bank (PDB) template (PDB identifier: 4M56) is superimposed on each of the models along with the relative ligand, and indicated in gray in all of the models. Helices are represented by round coiled arrows and β-sheets by flat arrows. Predicted domains are indicated as follows: domain B is encircled by a solid black line, domain B′ is encircled by a dotted black line, domain C is encircled by a dashed black line and domain A is the remaining un-circled portion of the structure.

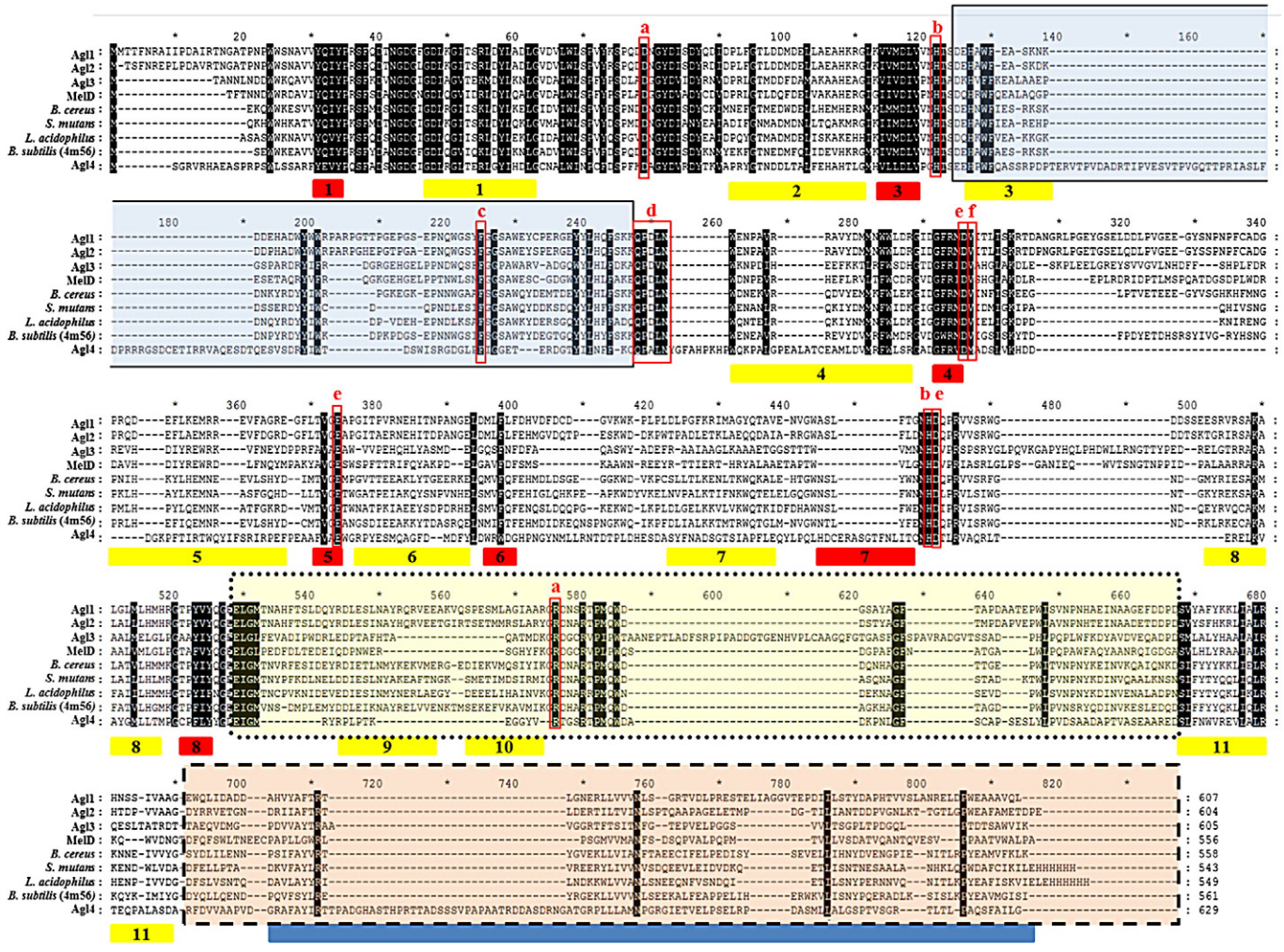


Fig. 6. Amino acid alignment of the four *B. breve* UCC2003-encoded GH13 α-glucosidases, the α-glucosidase-like protein Agl4, and four other GH13 glucosidases. Alignment of the four *B. breve* UCC2003-encoded GH13 α-glucosidases and the α-glucosidase-like protein Agl4, against four other GH13 glucosidases from *Bacillus cereus* (Watanabe et al., 2001), *Streptococcus mutans* (Hondoh et al., 2008), *Lactobacillus acidophilus* NCFM (Møller et al., 2012), and *Bacillus subtilis* (Schönert et al., 2006). Significant residues for the above four non *B. breve* UCC2003-encoded glucosidases had been previously experimentally determined. The deduced protein domains are indicated as follows: domain B is boxed by a solid black line and shaded in blue; domain B′ is boxed by a dotted black line and shaded in yellow; domain C is boxed by a dashed black line and shaded in orange; and domain A is the remaining unboxed portion of the alignment. Deduced structures are indicated as follows: TIM barrel strands are indicated by the filled red boxes under the alignment; α- helices are indicated by the filled yellow boxes under the alignment; and finally the anti-parallel β- pleated sheet is indicated by the filled blue box under the alignment. All predicted structures are numbered within the filled boxes in relation to their strand numbers. Highly conserved amino acid motifs across all nine are highlighted in black. Residues found to be conserved and with known functions in the four additional characterized glucosidases (refer to Results and Discussion) are highlighted in red and are indicated as follows: a. Asp79 and Arg576; b. His122 and His461; c. Phe225; d. The highly conserved QPDLN residues; e. Asp296, Glu374 and Asp462; and f. Val297. The numbering of all residues refers their relative position in the alignment.

encoded α-glucosidases and the α-glucosidase-like protein Agl4 (Fig. 6). However, within these two domains conserved regions can be identified between Agl1 and Agl2, and between Agl3 and MelD, consistent with their respective comparable enzymatic properties.

The close phylogenetic relationship observed for Agl1 and Agl2 is also reflected in a clear similarity in predicted protein structure (particularly in relation to the α-helices above the substrate binding site) as shown in Fig. 5 A and B, along with a high level of conservation in the amino acid alignment (particularly in relation to domains B and B′) in Fig. 6. This similarity at both sequence and predicted structural level is consistent with the observed biochemical characteristics (Pokusaeva et al., 2009). MelD and Agl3 are structurally more similar to each other than to Agl1, Agl2 or Agl4 (Fig. 5 A, B, C, D and E), especially within the area around the substrate binding site (upper side of the β-barrel).

An amino acid alignment (Fig. 6) was carried out for Agl1, Agl2, Agl3, Agl4 and MelD, along with characterized GH13 glucosidases from *Bacillus cereus* (Watanabe et al., 2001), *Streptococcus mutans* (Hondoh et al., 2008), *Bacillus subtilis* (Schönert et al., 2006), and *Lactobacillus acidophilus* NCFM (Møller et al., 2012). Notably, the highly conserved QPDLN residues, which are typical of the oligo-1,6-glucosidase subfamily (EC 3.2.1.10) (Oslancová and Janecek, 2002) are all conserved across Agl1, Agl2, Agl3 and MelD, although in Agl4 the Asp250 is replaced by Ala (Fig. 6d). A number of functionally significant residues are conserved across all five *B. breve* UCC2003-encoded proteins: Asp79 and Arg576 residues accomplish the recognition of the non-reducing end of the bound substrate (Hondoh et al., 2008; Fig. 6a); His122 and His461, and Phe225 are involved in substrate binding (Hondoh et al., 2008; Watanabe et al., 2001; Fig. 6b and c); and Asp296, Asp462 and Glu374 are all identified as active site residues (Hondoh et al., 2008; Møller et al., 2012; Watanabe et al., 2001; Fig. 6e).

Importantly, the acid/base catalyst Glu374 in Fig. 6, which has previously been identified as essential for the hydrolysis reaction by GH13 members (Møller et al., 2012), is fully conserved in the five analyzed *B. breve* proteins. This potential acid/base catalyst in Agl4 is followed by Trp375, whereas, the acid/base catalyst in the four active α-glucosidases (Agl1, Agl2, Agl3 and MelD) is followed by other relatively small residues (Ala, Ala, Ala and Ser, respectively), this difference may be (one of) the reason(s) for Agl4's very weak hydrolytic activity.

Moreover, Val297 (which has been identified as a specificity-determining signature residue towards glucosidic linkages within GH13 members), is conserved in Agl1, Agl2, Agl3 and MelD, yet appears to be replaced by Met297 in the Agl4 primary sequence (Yamamoto et al., 2004; Fig. 6f).

It is worth noting that Agl4 displays very little similarity to the other four enzymes within the regions associated with substrate interaction, perhaps indicating that an altered substrate interaction has contributed to the low α-glucosidic functionality (its hydrolytic activity is estimated to be at least 400-fold lower than that of Agl3). The differences observed in the multiple sequence alignment in Fig. 6 may have resulted in the absence of the two α-helices at the top of the substrate binding site on Agl4 (Fig. 5 E), potentially resulting in a altered and/or reduced ability of this protein in recruiting or retaining a substrate within its active site.

Agl4, is nonetheless, highly conserved among *B. breve* strains and various other bifidobacterial strains (where genome sequences are available). A multiple sequence alignment was carried out with Agl4 and 26 other bifidobacterial Agl4-homologs, which clustered together in the phylogenetic analysis (Figs. S2, 4). This alignment revealed that the Val297, which was found to be substituted for Met in Agl4, was similarly substituted in all other analyzed bifidobacterial Agl4-homologs (Fig. S2[a]). Fig. S2[b] also reveals that, similar to Agl4, the 26 bifidobacterial Agl4-homologs possess a Trp375 residue following the acid/base catalyst Glu374. Furthermore, the *agl4* gene has been shown to be transcriptionally upregulated under *in vivo* conditions (O'Connell Motherway et al., 2011), suggesting that Agl4 may nonetheless be of biological relevance to such bifidobacteria.

4. Conclusions

In order for *B. breve* UCC2003 to persist in the competitive environment of the human gastrointestinal tract, it has to be able to adapt to its surroundings. One major factor that affects growth and colonization of this gut commensal is the presence or absence of utilizable carbohydrate sources. The diversity and abundance of resistant carbohydrates is dependent on the diet of the human host and is therefore constantly in flux. Gut commensals, such as *B. breve* UCC2003, are therefore very adaptable to the transient nature of food sources in this particular, highly competitive environmental niche.

To conclude, *B. breve* UCC 2003 has the ability to produce four biologically relevant α-glucosidases (Agl1, Agl2, Agl3 and MelD), which exhibit differing substrate preferences that cover a range of possible α-glucosidic linkages. The presence of such a large variety of α-glucosidic activities within a single gut commensal adds to our understanding on specific metabolic adaptations and associated flexibility of (bifido) bacteria to the intestinal ecosystem. This abundance of α-glucosidases is likely to provide *B. breve* the metabolic agility to adapt to the transient nature of growth substrates in the GIT, thus contributing to gut colonization and persistence.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2016.02.014.

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