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RESEARCH ARTICLE

Human gut commensal bacterium *Ruminococcus* species FMB-CY1 completely degrades the granules of resistant starch

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Abstract Resistant starch (RS) in the diet reaches the large intestine and is fermented by the gut microbiota, providing beneficial effects on human health. The human gut bacterium FMB-CY1 was isolated and identified as a new species closest to *Ruminococcus bromii*. *Ruminococcus* sp. FMB-CY1 completely degraded RS including commercial RS types 2, 3, and 4, and generated glucose and maltose; however, it did not assimilate glucose. Genome analysis revealed 15 amylolytic enzymes (Amy) present in FMB-CY1. The evolutionary trees revealed that the Amys were well divided each other. All Amys (4, 9, 10, 12, and 16) containing cohesin and/or dockerin and scaffolding proteins known to be involved in constituting the amylosome, were identified. A new species of *Ruminococcus,* strain FMB-CY1, was considered to have the ability to form amylosomes for the degradation of RS. This new RS-degrading *Ruminococcus* species provides insights into the mechanism(s) underlying RS degradation in the human gut.

Keywords Amylosome · Gut microbiota · Resistant starch · *Ruminococcus* · Starch granule

Introduction

Starch consists of simple glucose molecules linked by α-1,4 and α-1,6-glycosidic bonds. It is composed of amylose and amylopectin and is considered a major component of the human diet, accounting for over 25% of a typical individual's daily calories (Bello-Perez et al., 2020). Within the digestive tract of humans, there are a series of starch-degrading enzymes, including amylase, isoamylase, and amyloglucosidase. These enzymes help in degrading

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and assimilating starch in the digestive tract for use as an energy source (Bello-Perez et al., 2020). However, a type of starch called resistant starch (RS) is known to be resistant to human digestive enzymes. Without being degraded in the early digestive tract, RS is able to reach the colon and is used as a source of energy by gut microbiota (Fuentes-Zaragoza et al., 2011). According to their physical structures, RS is generally divided into five groups: RS1, physically inaccessible; RS2, native granular structure; RS3, retrogradation; RS4, chemically modified; and RS5, amylose–lipid complexes (Raigond et al., 2015). Recently, numerous studies have suggested that the intake of RS has many positive roles in improving human health. For instance, RS is known to lower the risk of insulin resistance and leptin resistance by preventing rapid glucose release due to its lower digestibility (Maziarz et al., 2017; Sandberg et al., 2017; Shaikh et al., 2019). In addition, RS is fermented by intrinsic gut microbiota in the large intestine and is transformed into short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate. These small molecules are known to play a role in maintaining human health by regulating luminal pH, providing fuel for epithelial cells, and influencing mucosal immune function (Blaak et al., 2020).

Although many amylolytic gut bacteria, including *Bacteroides* spp., *Eubacterium* spp., and *Bifidobacterium* spp., have been reported, the number of bacteria that can degrade RS remains scarce. *Ruminococcus bromii* and *B. adolescentis* (and other related *Bifidobacterium* species) are the two main species with RS-degrading capability found in the human gut microbiota (DeMartino and Cockburn, 2020). *R. bromii*, a strict anaerobe, is important for the gut microbial community related to RS. One study found that the abundance of *R. bromii* increased markedly in fecal samples from human volunteers who switched to diets high in RS (Flint, 2012; La Reau et al., 2016). Furthermore, *R. bromii* has been defined as a keystone species because of its excellent RS degradation ability and its ability to feed other gut microbiota via RS fermentation products in the large intestine (Ze et al., 2012). Ze et al. (2015) also reported a distinctive degradation mechanism of *R. bromii* L2-63, in which extracellular protein complexes called amylosomes were involved. Amylosomes are unique multienzyme complexes similar to cellulosomes and are found in various cellulolytic microorganisms. In amylosome complexes, several extracellular amylases are assembled through interactions between dockerin and cohesin modules present in enzymes and structural proteins, similar to cellulosomes. In contrast, Jung et al. (2018, 2019) identified two strong RS-degrading bacteria, *B. choerinum* FMB-1, from the rumen fluids of Korean native cattle (*Bos taurus coreanae*) and *B. adolescentis* P2P3 from human feces. Interestingly, in these strains, the formation and precipitation of insoluble high-amylose corn starch (HACS) granule clusters were observed before the HACS granules were decomposed by these bacteria. This unusual characteristic may be explained by cell surface-anchored protein complexes in which starch-binding domains and RS-degrading enzymes are involved. This mechanism is strikingly different from that of the amylosome of *R. bromii* L2-63 (Ze et al., 2015).

To date, only two species of gut bacteria, so-called *R. bromii* and *B. adolescentis,* have been found to be involved in primary RS degradation in the human gut; however, it is likely that others exist (DeMartino and Cockburn, 2020). In the present study, we isolated unidentified strains that are capable of utilizing RS as a primary degrader from human fecal samples. As a result, the new *Ruminococcus* species strain FMB-CY1, which was able to degrade HACS superior to other previously reported gut bacteria, was isolated (Jung et al., 2019; Ze et al., 2012). Whole-genome sequence analysis of *Ruminococcus* sp. FMB-CY1 was performed, and the uniqueness of its gene content in relation to RS degradation was investigated.

Materials and methods

Starches substrates and other reagent

As resistant starch (RS) substrates, S4126 and S4180 (Sigma-Aldrich, St Louis, MO, USA) were employed. S4126 is an unmodified regular corn starch containing approximately 73% amylopectin and 27% amylose, whereas S4180 is an unmodified high amylose corn starch (HACS) classified as RS2 containing 70% amylose. The food-grade commercial RS, Hi-Maize (HM) 260, HM 958, Novelose 330, Versafibe (VF) 1490, and VF 2470 were kindly provided by Ingredion (Westchester, IL, USA). In the case of commercial RS2 type starches (HM 260 and 958), HM 260 was prepared from Hylon VII, which is a commercial HACS (70% amylose) (Le Leu et al., 2009) whereas Hi-maize 958 is unmodified food-grade RS2 containing 61.8% of RS (Le Leu et al., 2005). RS3 type starch (NV 330) is a retrograded starch generated from hydrolyzed high-amylose corn starch products. VF 1490 and 2470 (RS4 type starch) were derived from potato starch and HACS, respectively. VF 1490 is a distarch phosphate modified using phosphorus oxychloride, and VF 2470 is produced by hydrolysis and heat treatment of high-amylose maize starch.

Mono-, di-, and tri-saccharides (glucose, fructose, maltose, sucrose, cellobiose, maltotriose, and panose) and polysaccharides (soluble starch, pectin, pullulan, glycogen, and carboxymethyl cellulose) were purchased from Sigma-Aldrich and Megazyme (Bray, Ireland). The medium,

chopped meat (CM) broth, was obtained from MBcell (Seoul, Korea), and all other medium supplements for human fecal bacteria were purchased from Sigma-Aldrich. All other chemicals used in this study were of analytical grade.

Growth media for human fecal bacteria

CM broth containing 0.1% (v/v) trace mineral and vitamin solutions (CMTV) was used to isolate and incubate anaerobic human fecal bacteria. The CM broth consisted of (per 100 mL) peptone (3.0 g), yeast extract (0.5 g), dipotassium phosphate (0.5 g), L-cysteine (0.05 g), and resazurin (0.1 mg). The trace mineral solution (per liter) consisted of nitrilotriacetic acid (1.5 g), $MgSO_4{\cdot}7H_2O$ (3 g), $MnSO_4$ (0.5 g), NaCl (1 g), $FeSO_4{\cdot}7H_2O$ (0.1 g), $CoSO_4{\cdot}7H_2O$ (0.18 g), $CaCl_2{\cdot}2H_2O$ (0.1 g), $ZnSO_4{\cdot}7H_2O$ (0.18 g), $CuSO_4{\cdot}5H_2O$ (0.01 g), $KAl(SO_4)_2{\cdot}12H_2O$ (0.02 g), H_3BO_3 (0.01 g), $Na_2MoO_4{\cdot}2H_2O$ (0.01 g), and $NiCl_2{\cdot}6H_2O$ (0.025 g). The vitamin solution (per liter) consisted of biotin (2 mg), folic acid (2 mg), pyridoxine–HCl (10 mg), thiamine-HCl·$2H_2O$ (5 mg), riboflavin (5 mg), nicotinic acid (5 mg), D-pantothenic acid (5 mg), vitamin B12 (0.1 mg), 4-aminobenzoic acid (5 mg), and lipoic acid (5 mg). The CMTV medium was dissolved in serum vials and autoclaved. Starch substrates were sterilized separately from the medium to prevent gelatinization. Then, sterilized starch substrates were mixed with a sterilized medium, followed by flushing with 99.5% CO_2 gas using a 0.2-μm filter.

Isolation of RS-degrading human fecal bacteria

Fecal samples were obtained from a healthy Korean male adult (age 53 years) with no history of gastrointestinal diseases and who had not taken any antibiotics in the previous year. The donors consumed a regular diet for four weeks. The human fecal sample was quickly placed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) filled with 5% CO_2 and 95% N_2 gas within five minutes. The human fecal sample was diluted ten-fold (5 g/50 mL) with a sterile PBS solution (pH 7.4), followed by homogenization for inoculation.

RS-degrading human fecal bacteria were isolated in four steps: (1) Preparation of agar medium: Modified CMTV medium containing 0.5% soluble starch was autoclaved and mixed with sterile S4126. The mixed medium was poured with gentle pipetting to prevent bubble formation and starch precipitation. (2) Spreading of fecal slurry: 100 μL of prepared fecal slurry was spread on CMTV agar medium and incubated under anaerobic conditions at 37 °C for 5–6 days. (3) Selection of raw corn starch-degrading strains: Colonies exhibiting a clear zone around the colony, that is, decomposing raw corn starch, were selected. (4) Identification of RS-degradation: Selected strains were inoculated in CMTV broth containing 0.5% S4180. After incubating for 3 days at 37 °C with 30 rotations per minute, residual RS was measured to confirm the degradation of RS.

Identification and phylogenetic analysis of RS-degrading human fecal bacteria

Genomic DNA was extracted using a stool DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 16S rRNA gene amplification was performed using universal primers (27F and 1492R) and KOD polymerase (Toyobo, Osaka, Japan). The PCR conditions included an initial denaturation at 95 °C for 5 min; 30 cycles consisting of 30 s at 95 °C, 30 s at 55 °C, and 1.5 min at 72 °C; and a final extension at 72 °C for 10 min. The amplified fragments were sequenced and identified using the EzBioCloud database ver. 2021.04.13 (https://www.ezbiocloud.net/). The phylogenetic tree was constructed using MEGA X software (Kumar et al., 2018). The 16S rRNA gene sequence of *Ruminococcus* sp. FMB-CY1 was deposited in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession number MZ573208. The average nucleotide identity (ANI) values were computed using OrthoANI with the original ANI option (Lee et al., 2016). *Ruminococcus* sp. FMB-CY1 isolated in this study was deposited in the Korean Agricultural Culture Collection (KACC, Wanju, Korea) under collection number KACC 22405.

RS degradation ability and utilization of other carbohydrates

Ruminococcus sp. FMB CY1 were anaerobically cultivated in serum vials containing CMTV broth prepared as in the 'Growth media' section. As a seed culture, *Ruminococcus* sp. FMB-CY1 was anaerobically pre-cultured in 20 mL of CMTV broth containing 0.5% maltose at 37 °C until an optical density at 600 nm of 0.6 was reached. The seed culture (200 μL) was inoculated into 20 mL of CMTV broth containing 0.5% (w/v) S4180. The inoculation and subculture process were performed in anaerobic chamber (Coy Laboratory Products) filled with 5% CO_2 and 95% N_2 gas. After incubating at 37 °C with 30 rotations per minute, residual RS and released reducing sugars were measured every 4 h to confirm the degradation of RS granules. The seed culture (200 μL) was inoculated into 20 mL of CMTV broth containing 0.3% (w/v) other carbohydrate substrates (mono-, di-, tri-, and polysaccharides) to confirm the utilization of other carbohydrates. After incubation under the

same conditions, growth was measured spectrophotometrically at an optical density (OD) of 600 nm.

Scanning electron microscopy (SEM)

The culture samples of RS and human fecal bacteria were cautiously dried with nitrogen gas and placed on a carbon tape over a microscope slide to be coated with gold under vacuum. The images were obtained using a Tabletop Microscope TM 3000 (Hitachi, Japan). The particle size was determined from a micrograph with a scale bar of 30 μm (3000×).

Quantification of residual starch and reducing sugars

RS utilization was measured using all residual carbohydrates via the phenol–sulfuric acid method (Masuko et al., 2005). Briefly, a culture sample (100 μL) was mixed with a 5% phenol solution (200 μL). Eight-hundred microliters of 99% sulfuric acid was added, and the reaction was gently mixed and incubated at 30 °C for 20 min. In contrast, the RS degradation ability was measured using only the remaining insoluble starch. First, insoluble starch in the culture was isolated by centrifugation. Next, the pellet was washed twice and resuspended in distilled water. Then, 100 μL of the suspension solution was assigned to the sample. After the addition of 5% phenol solution (200 μL), the sample was treated as described above.

Reducing sugars in the culture was measured using the dinitrosalicylic acid (DNS) method (Miller, 1959). Briefly, 100 μL of cell-free supernatant was mixed with 300 μL of DNS solution, followed by boiling for 5 min. The absorbance of the phenol–sulfuric acid and DNS reactant was measured at 555 nm using an iMark microplate reader (BioRad, Hercules, CA, USA). The experiments were repeated in triplicate.

Thin layer chromatography (TLC) analysis

TLC silica gel 60G F25425 glass plates (Merck Millipore, Billerica, MA, USA) were activated for 5 min at 110 °C. One microliter of culture supernatant was loaded twice onto TLC plates and developed with developing solution composed of 1-butanol: acetic acid: water at a 5:2:3 (v/v/v) ratio. The plate was dried and developed rapidly by dipping in a solution containing 0.3% (w/v) 1-naphthol and 5% (v/v) sulfuric acid in methanol. The plate was dried and placed in an oven at 110 °C for 5 min.

Genome sequencing and gene analysis

The genomic DNA of *Ruminococcus* sp. FMB-CY1 was extracted using a QIAamp DNA Mini Kit (Qiagen). The extracted genomic DNA was quantified using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and a Qubit 2.0 fluorometer (Thermo Fisher Scientific). The whole genome of the strain was sequenced using a PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA) sequencing platform. The sequenced reads were assembled using HGAP 3.0 (Chin et al., 2013) with a 2-Mb expected genome size. Chromosome topology was drawn using DNAPlotter (Carver et al., 2008). Annotation of the protein-coding genes was performed using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016).

Functional classification of the protein-coding genes of *Ruminococcus* sp. FMB-CY1 was predicted using BlastP search with two criteria: an *e*-value of 1e − 5 and a minimum coverage of 50% from the NCBI Clusters of Orthologous Groups (COG) database. Each gene was classified into COG categories, and unassigned genes were described as "not assigned." Putative antimicrobial resistance genes were predicted using the MEGARes database (Doster et al., 2019) with BlastN. The Blast hits were screened using the following criteria: > 80% identity, > 50% coverage, and a 40 bp minimum alignment length. The genome sequence was uploaded to the NCBI Genome Database under accession number CP084034 (https://www.ncbi.nlm.nih.gov/genome/).

Carbohydrate-active enzymes were detected using dbCAN 2 with HMMER, DIAMOND, and Hotpep databases (Zhang et al., 2018), and amylolytic enzymes and proteins were organized. The prediction of signal peptides (Sec- and Tat-secretion) of enzymes and proteins was performed using the CBS-SignalP 5.0 server (Armenteros et al., 2019). The conserved domains of enzymes and proteins were defined using the NCBI-CD search (Marchler-Bauer et al., 2017). The corresponding proteins against *R. bromii* species (ATCC 27255 and L2-63) were analyzed by alignment using NCBI BlastP (Johnson et al., 2008). Multiple alignment of the corresponding proteins was performed using EBI-Clustal Omega (Sievers et al., 2011). Evolutionary trees were constructed using MEGA X software (Kumar et al., 2018).

Results and discussion

Isolation and identification of RS-degrading *Ruminococcus* sp. FMB-CY1

An RS-degrading obligate anaerobic gut bacterium, strain FMB-CY1, was isolated from the human fecal microbiota

of a healthy 53-years old male. The strain FMB-CY1 showed a clear zone around the colony on the raw corn starch-containing agar plate (data not shown) and the aggregation of RS granules in CMTV broth, indicating that this strain had the ability to decompose raw starch and RS granules. The granules remained intact at the initial stage (Fig. 1a). However, after 14 h, the RS granules aggregated to form a granule cluster (Fig. 1b). These granule clusters gradually disappeared until 36 h, as all the RS clusters were degraded (Fig. 1c). The aggregation of RS granules by an RS-degrading bacterium was previously observed in *B. adolescentis* P2P3 (Jung et al., 2020).

As a result of identification (Fig. 2a), the 16S rRNA sequence of the strain FMB-CY1 was mostly closest to *R. bromii* ATCC 27255 ^{T} (96.74%), followed by *Clostridium jeddahense* JCD^{T} (91.23%), *Hydrogeniiclostidium mannosilyticum* ASD2818^{T} (91.08%), *Caproiciproducens galactitolivorans* BS-1 ^{T} (91.05%), *Ca. fermentans* EA1^{T} (90.76%) and *Cl. sporosphaeroides* DSM 1294 ^{T} (90.52%), and *R. champanellensis* 18P13^{T} (89.85%). Based on the phylogenetic tree, the strain FMB-CY1 was bound to a clade with *R. bromii* ATCC 27255 ^{T}. In terms of the average nucleotide identity (ANI) values (Fig. 2b), strain FMB-CY1 showed a high similarity to *R. bromii* ATCC 27255 ^{T} (75.15%), but was far below 94%. Therefore, strain FMB-CY1 is proposed as a new species with a high possibility of belonging to the *genus Ruminococcus*. Therefore, the isolated strain FMB-CY1 was designated as *Ruminococcus* sp. FMB-CY1.

RS degradation ability of *Ruminococcus* sp. FMB-CY1

The degradation of RS Type 2 starch (non-gelatinized high-amylose corn starch; S4180) and the resulting reducing sugar products were measured over time by incubating *Ruminococcus* sp. FMB-CY1 (Fig. 3). S4180 was degraded slowly until 8 h after inoculation, then rapidly decomposed until 20 h, and almost all S4180 disappeared within 30 h (Fig. 3a). The amount of reducing sugars increased until 24 h when RS degradation was nearly complete, and thereafter slightly decreased (Fig. 3b). It was thought that the assimilation rate of the sugars released from S4180 by bacteria overcame the degradation rate of RS at 24 h. In addition, an increase in sugars, such as glucose, maltose, and maltotriose, during the growth of *Ruminococcus* sp. FMB-CY1 expression was confirmed by TLC analysis (Fig. 3b). Similar to S4180, many commercial RS Types 2, 3, and 4, such as HM 260, NV 330, and VF 2478, were also completely degraded, except for VF1490, which was processed from potato starch (Fig. S1). Only 70% of VF1490 was degraded by *Ruminococcus* sp. FMB-CY1. Hence, raw potato starch is considered to be more difficult to decompose by this strain than other RS.

Utilization of various carbohydrates by *Ruminococcus* sp. FMB-CY1

The growth of *Ruminococcus* sp. FMB-CY1 was measured to evaluate the carbon source utilization ability using several carbohydrates as the sole substrate. With mono-, di-, and tri-saccharides (Fig. 4a), *Ruminococcus* sp. FMB-CY1 grew well under maltose, fructose, and maltotriose. However, glucose, sucrose, cellobiose, and panose could not be used as the sole carbon sources. In the case of polysaccharides (Fig. 4b), soluble starch, pullulan, and glycogen were considered as easily accessible carbon sources for this bacterium. However, pectin and CM cellulose were not assimilated by *Ruminococcus* sp. FMB-CY1. Although the strain FMB-CY1 has high resistant starch degradation activity, it did not utilize glucose as a single carbon source, but instead used fructose and maltose preferentially (Bren et al., 2016). This observation suggested that there is no functional glucose transport/uptake system in *Ruminococcus* sp. FMB-CY1. Previously, three

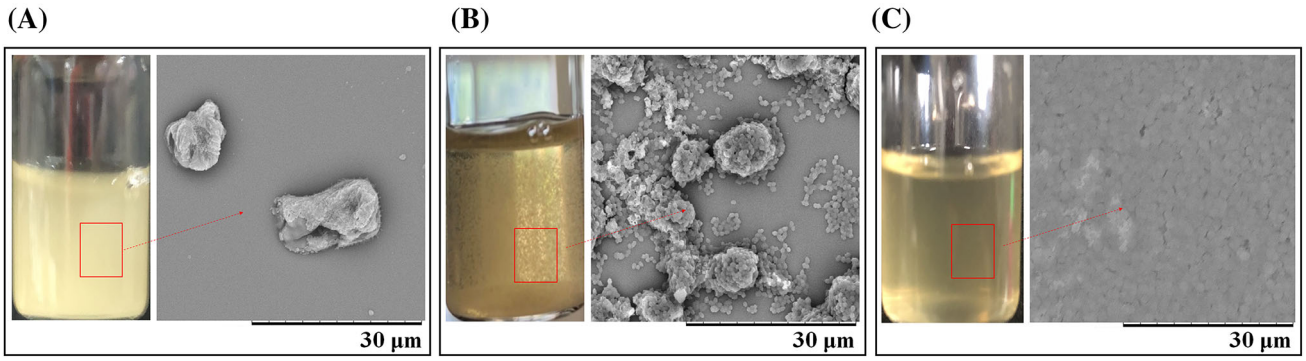

Fig. 1 Scanning electron microscopy during degradation of RS granules by *Ruminococcus* sp. FMB-CY1. (**A**) Dispersed RS granule at 0 h of incubation. (**B**) Granule cluster formation at 14 h of incubation. (**C**) Solely degraded RS at 36 h of incubation

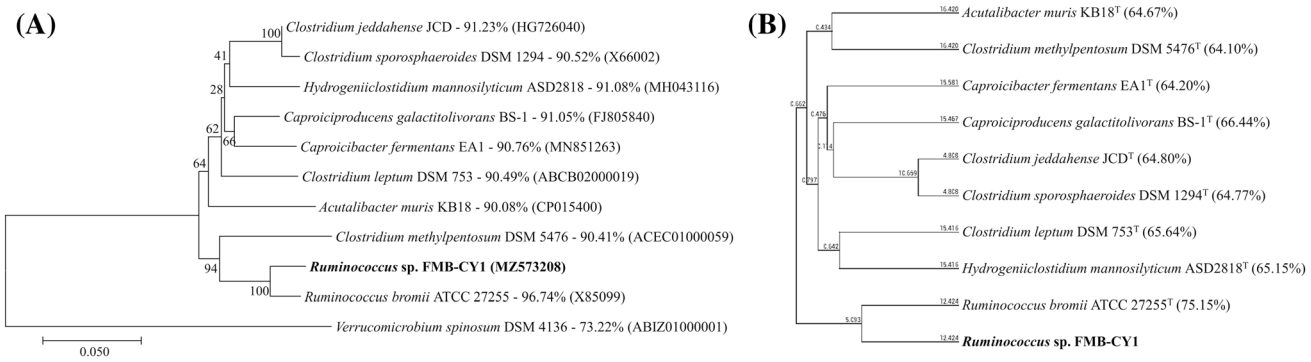

Fig. 2 Identification of the human gut bacterium *Ruminococcus* sp. FMB-CY1. (**A**) Phylogenetic tree of 16S rRNA gene sequences of strain FMB-CY1. The type strains of closely related species based on the neighbor-joining algorithm applied with bootstrap replications of 1000 datasets. Scale bar, 0.05 accumulated changes per nucleotide. (**B**) Average nucleotide identity tree of whole genome of strain FMB-CY1. The type strains based on original ANI method chopped to 1020 bp. The numbers on the tree represent the distance between species

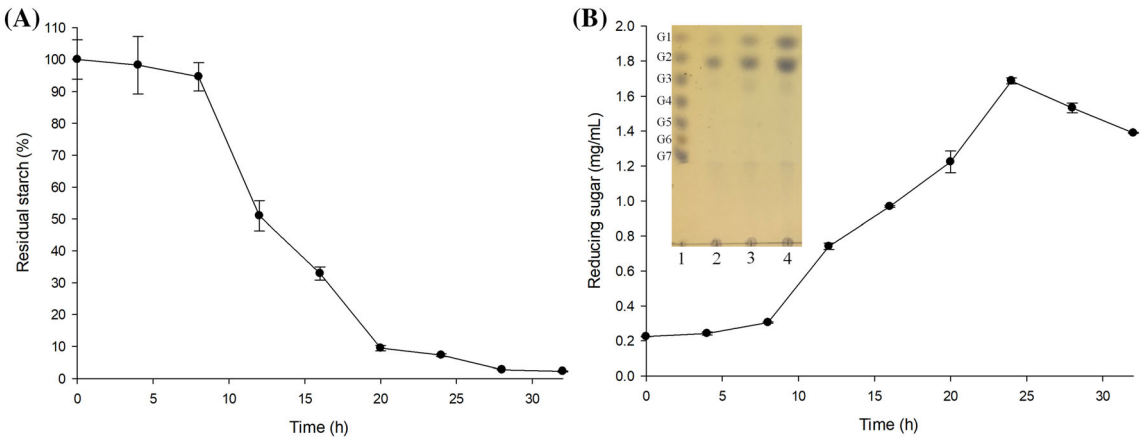

Fig. 3 RS degradation ability and reducing sugar release incubating *Ruminococcus* sp. FMB-CY1. The time course profiles of (**A**) the quantification of residual insoluble starch during incubation criteria on 0 h as control, and (**B**) the quantification of reducing sugars releasing from RS granule decomposition and TLC analysis of supernatant of culture medium. Lane 1 to 5: G1-G7 standard, incubated to 0 h, 24, and 48 h, respectively

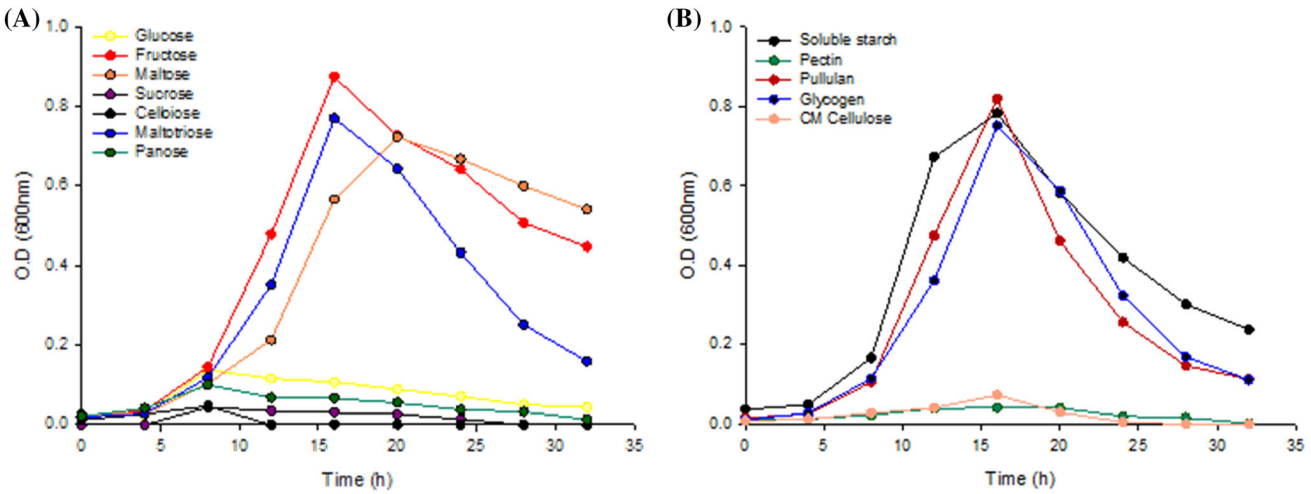

Fig. 4 Growth of *Ruminococcus* sp. FMB-CY1 on various carbohydrate sources. The growth curves on CMTV broth containing 0.3% of the (**A**) mono-, di-, tri-saccharides, and (**B**) polysaccharides

Table 1 Enzymes and proteins involved in the degradation of starch substrates of *Ruminococcus* sp. FMB-CY1

Protein ID	Presumed function	Length (aa)	Signal peptide	Carbohydrate binding modules	Anchoring/binding region	Enzyme Class	Corresponding protein (ID/cover/identity)		Assignment
							R. bromii ATCC 27255	*R. bromii* L2-63	
FMBCY1_000046	Cyclomaltodextrinase	631	–	–	–	GH13_39	PKD32690.1 (98%/73.72%)	SPE91311.1 (98%/73.63%)	Amy 3
FMBCY1_000250	Starch-binding protein	737	[A]Sec/SPI	CBM26, CBM74	[C]Doc	–	PKD28238.1 (100%/68.16%)	SPE92126.1 (99%/69.41%)	[E]NA1
FMBCY1_000353	1,4-α-Glucan branching protein	697	–	CBM48	–	GH13_9	PKD27046.1 (91%/83.65%)	SPE91075.1 (91%/83.49%)	Amy 13
FMBCY1_000428	1,4-α-Glucan branching protein	658	–	–	–	GH13_9	PKD27747.1 (96%/67.72%)	SPE91806.1 (97%/68.23%)	Amy 14
FMBCY1_000504	Pullulanase, type I	1236	Sec/SPI	CBM26 × 2, CBM48	Doc	GH13_14	PKD26628.1 (100%/64.47%)	SPE91787.1 (100%/64.71%)	Amy 10
FMBCY1_000688	α-Amylase	679	[B]Sec/SPII	CBM26, CBM74	–	GH13_28	PKD28240.1 (84%/70.66%)	SPE92124.1 (45%/69.28%)	Amy 17
FMBCY1_000714	Starch-binding protein	521	Sec/SPI	CBM26 × 2	Doc	–	PKD32717.1 (99%/53.35%)	SPE91138.1 (99%/55.27%)	NA2
FMBCY1_000715	α-Amylase	800	Sec/SPI	CBM26	–	GH13_42	PKD32718.1 (100%/74.01%)	SPE91137.1 (100%/74.13%)	Amy 1
FMBCY1_000734	Cyclomaltodextrinase	549	–	–	–	GH13_20	PKD28303.1 (99%/74.51%)	SPE92578.1 (99%/74.25%)	Amy 8
FMBCY1_001182	α-Amylase	1038	Sec/SPI	CBM26	Doc	GH13_28	PKD29375.1 (99%/59.55%)	SPE92705.1 (99%/58.82%)	Amy 9
FMBCY1_001538	Pullulanase, type I	1056	Sec/SPI	CBM26	Doc	GH13_14	PKD30566.1 (99%/64.02%)	SPE92040.1 (99%/63.81%)	Amy 12
FMBCY1_001539	Pullulanase, type I	957	Sec/SPI	CBM48	LPXTG	GH13_14	PKD30567.1 (98%/67.37%)	SPE92039.1 (98%/67.80%)	Amy 11
FMBCY1_001797	Cyclomaltodextrinase	753	Sec/SPI	CBM26	–	GH13_42	PKD32129.1 (100%/78.78%)	SPE91197.1 (100%/78.12%)	Amy 2
FMBCY1_001956	α-Amylase	557	Sec/SPII	–	–	GH13_36	PKD27034.1 (99%/63.84%)	SPE91476.1 (99%/63.84%)	Amy 5
FMBCY1_002110	α-Amylase	878	Sec/SPI	CBM26	Doc	GH13_28	PKD32488.1 (99%/57.17%)	SPE92332.1 (99%/57.77%)	Amy 16
FMBCY1_002193	α-Amylase/α-Glucosidase	511	–	–	–	GH13_37	PKD26944.1 (100%/79.06%)	SPE91558.1 (100%/79.45%)	Amy 6
FMBCY1_002258	α-Amylase	1648	Sec/SPI	CBM26	[D]Coh	GH13_19	PKD32588.1 (71%/56.73%)	SPE91379.1 (70%/56.85%)	Amy 4
FMBCY1_002260	α-Amylase	1233	Sec/SPI	CBM26	[D]Coh/Doc	GH13_19	PKD32588.1 (100%/56.97%)	SPE91379.1 (100%/57.09%)	Amy 4
FMBCY1_002303	Glycogen phosphorylase	789	–	–	–	GT35	PKD26876.1 (99%/87.82%)	SPE91261.1 (99%/87.94%)	NA3
FMBCY1_002304	4-α-Glucanotransferase	495	–	–	–	GH77	PKD26877.1 (100%/91.11%)	SPE91262.1 (100%/90.91%)	NA4

[A]Sec/SPI: "standard" secretory signal peptides transported by the Sec translocon and cleaved by Signal Peptidase I, [B]Sec/SPII: lipoprotein signal peptides transported by the Sec translocon and cleaved by Signal Peptidase II., [C]Dockerin, [D]Cohesin, [E]NA: Not assigned

R. bromii strains (L2-63, 5AMG, and ATCC 27255) isolated from human feces were also found to grow on fructose, galactose, and maltose, but not glucose (Mukhopadhya et al., 2018). The fructose-specific phosphotransferase gene cluster was inherent in these strains, and it has been suggested that maltose is metabolized by a phosphorylase/glucanotransferase cycle similar to that of *E. coli* (Boos and Shuman, 1998; Mukhopadhya et al., 2018). Likewise, the fructose-specific phosphotransferase gene cluster and ABC-type maltose transport system were present in *Ruminococcus* sp. FMB-CY1 (data not shown). In addition, the inability to absorb glucose by *Ruminococcus* sp. FMB-CY1 may be beneficial for promoting the growth of other commensal gut bacteria.

In addition, the number of bacteria rapidly decreased without a stationary phase after growth up to OD 0.8–0.9 for all carbon source conditions. This is an interesting result because the stationary phase is maintained for a certain period of time in most bacteria. This characteristic of rapid death is believed to be related to the autolytic activity of *Ruminococcus* sp. FMB-CY1 (Molinero et al., 2021). This indicates that the autolysis mechanism may be turned on when the cell density reaches a certain point.

Genome analysis of *Ruminococcus* sp. FMB-CY1

The complete genome of *Ruminococcus* sp. FMB-CY1 was composed of a 2,375,995-bp single chromosome with a 38.98% GC content (Fig. S2 and Table S1). A total of 2324 genes were identified, including 2245 protein-coding genes, 79 RNA genes, and 35 pseudogenes. The genome contained five, four, and four sets of full-length 5S, 16S, and 23S rRNA genes, 61 tRNA genes, and five non-coding RNA genes. One CRISPR array was identified at position 378,712–384,474 of the genome. *Ruminococcus* sp. FMB-CY1 is the first species with a complete genome sequence compared to *R. bromii* as the most related species. As shown in Table S1, the other genome sequences (ATCC 27225 and L2-63) were not assembled completely, and 74 and 40 contigs were reported, respectively. Genome sizes of *R. bromii* ATCC 27225 and *R. bromii* L2-63 were 2.15 Mb, and 2.24 Mb, respectively, similar to that of FMB-CY1. Likewise, the GC content of these strains was almost similar to that of FMB-CY1 by 40.7% and 41.4%, respectively.

Among the 2324 protein-coding genes of strain FMB-CY1, 1933 genes were assigned as defined proteins, whereas 391 genes were designated as hypothetical proteins (Table S2). The percentage of proteins with unknown functions, including "general function prediction only (R)," "function unknown (S)," and "not assigned," was 27.3%. One antimicrobial resistance gene (FMB-CY1_001903) was found in the FMB-CY1 genome (Table S3). The biological mechanism of FMB-CY1_001903 was annotated as an aminoglycoside *O*-nucleotidyltransferase based on the definition of the MEGARes. Bacteria typically develop their antibiotic resistance mechanisms by gene(s) encoded in chromosome or plasmid. Antibiotic resistance can be expressed by a single gene or multiple genes requiring complex relationship. In fact, the gene corresponding to an aminoglycoside *O*-nucleotidyltransferase was not found by Blast alignment against the genomes of *R. bromii* strains ATCC 27255 and L2-63.

Putative amylolytic enzymes in *Ruminococcus* sp. FMB-CY1

When raw granular starch is consumed by bacteria, starch granules cannot be transported into the cell because of their size and structure. Starch granules are resistant to common starch-hydrolyzing enzymes; hence, specific granule-degrading enzymes are required. The enzymes and proteins of *Ruminococcus* sp. FMB-CY1, which is considered to directly or indirectly affect RS degradation, was determined. Eighteen genes encoding amylolytic enzymes active on α-glucan substrates and two genes possibly translating to the starch-binding protein (FMBCY1_000250 and FMBCY1_000714) were detected (Table 1). Most enzymes belonged to the glycosyl hydrolase (GH) family 13 and were predicted to function as cyclomaltodextrinases, 1,4-α-glucan branching enzymes, pullulanases, and α-amylases. In addition, one glycogen phosphorylase and one 4-α-glucanotransferase belonging to the glycosyltransferase (GT) 35 and GH77 family, respectively, were identified. Interestingly, most of the aforementioned enzymes and proteins had a signal peptide, and as such were considered to be localized extracellularly. Moreover, all proteins with signal peptides also possessed carbohydrate-binding modules (CBM) in the protein, except for one (FMBCY1_001956). In fact, CBM is known to be involved in binding to polysaccharides and carrying appended catalytic domains into intimate contact with target substrates (Blake et al., 2006). Therefore, these enzymes and proteins may be secreted outside the cell by signal peptides, playing a crucial role in decomposing relatively large insoluble starch granules at the cell surface.

Mukhopadhya et al. (2018) reported that RS-degrading *R. bromii* L2-63 could form a cell-surface enzyme complex called amylosome by the cohesin and dockerin domains, and 17 amylolytic enzymes (assigned as Amy 1 to 17) were proposed to be involved in the degradation of RS. The enzymes corresponding to the 17 Amys of RS-degrading *R. bromii* strains ATCC 27255 and L2-63 were also present in the genome of *Ruminococcus* sp. FMB-CY1, except for Amy 7 and 15 (predicted to be α-amylases and glycogen

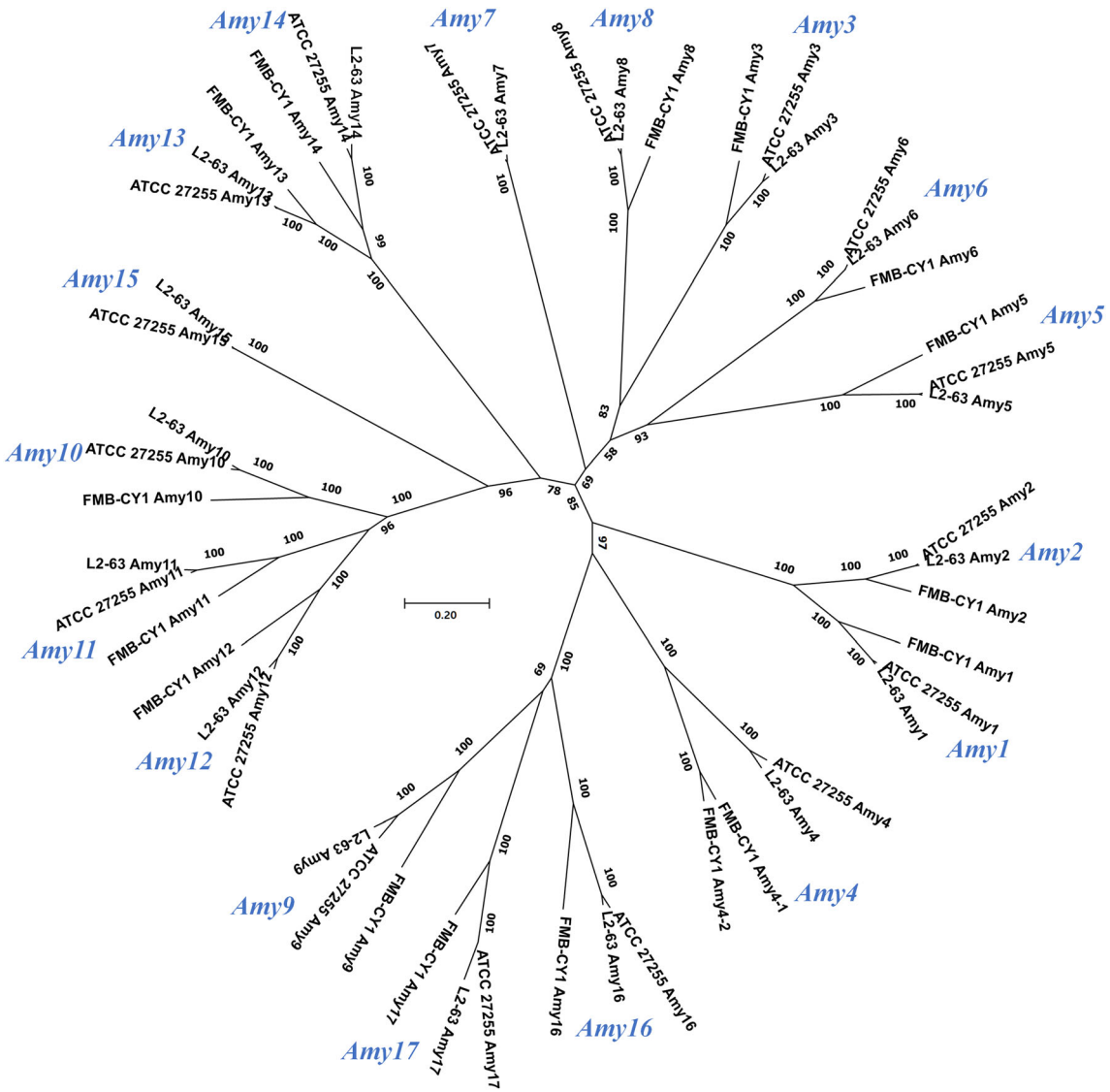

Fig. 5 Evolutionary trees including Amys 1 to 17 characterized from the RS-degrading *Ruminococcus* strains. The applied strains are *Ruminococcus* sp. FMB-CY1, *R. bromii* ATCC 27255, and *R. bromii* L2-63. The minimum evolution algorithm with bootstrap replications of 1000 datasets was applied. Scale bar, 0.05 accumulated changes per amino acids

debranching enzymes, respectively) (Table 1 and Fig. 5). In addition, four unassigned enzymes or proteins were also detected in the genome of FMB-CY1, which were also present in the *R. bromii* strains ATCC 27255 and L2-63. The sequence coverage of the corresponding Amys of FMB-CY1 to other *R. bromii* strains was 99%, and the identities ranged from 56 to 91%. The evolutionary trees, including Amys 1 to 17, characterized from the RS-degrading *Ruminococcus* strain (FMB-CY1, ATCC 27255, and L2-63) revealed that the same protein group of Amys was well divided and close to each other. These results suggest that these Amy enzymes may be functionally analogous in the RS-degrading *Ruminococcus* strain (Fig. 5). The corresponding Amys of *Ruminococcus* sp. FMB-CY1 also possessed a dockerin or cohesin domain. Regarding Amy 4, two proteins (FMBCY1_002258 and FMBCY1_002260) were observed, and both α-amylases had signal peptides, CBM26, and cohesin domains. However, the dockerin domain was only found in FMBCY1_002260.

Five scaffolding proteins (Sca 1, 2, 3, 4, and 5) involved in amylosome formation have been previously reported (Mukhopadhya et al., 2018). In *Ruminococcus* sp. FMB-CY1, five proteins containing a possible cohesin domain were also detected, although the sequence identities were rather low compared to those of *R. bromii* L2-63 (28% to 44%) (Table S4). Among them, two proteins (FMBCY1_002258 and FMBCY1_002260), corresponding

to Amy 4 in *R. bromii* L2-63, contained α-amylase catalytic domains. Although detailed studies on amylosomes in *Ruminococcus* sp. FMB-CY1 are required, *Ruminococcus* sp. FMB-CY1 appears to degrade RS by forming cell-surface amylosomes through the corresponding Amys and scaffolding proteins.

To date, only one species of *Ruminococcus* (*R. bromii*) has been reported to degrade RS as a primary degrader in the human gut. In this study, human gut-originated *Ruminococcus* sp. FMB-CY1, which was able to completely degrade RS, was isolated as a new species of the genus *Ruminococcus*. The analysis of its RS degradation characteristics and starch-degrading enzymes provides fundamental insights into the degradation of RS by *Ruminococcus* species, a keystone species in the human gut. In conclusion, the findings presented on this new RS-degrading *Ruminococcus* species are essential as it is a key species for RS degradation in the human gut, and shed light on the metabolic fate of RS.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of human derivatives were followed. The study of human derivatives was approved by the Institutional Review Board of Kyung Hee University (KHSIRB-17–004).

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