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Review Article

Functions, structures and applications of cellobiose 2-epimerase and glycoside hydrolase family 130 mannoside phosphorylases

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Abbreviations: AGE, *N*-acylglucosamine 2-epimerase; AKI, aldose-ketose isomerase; *Bf*MGP, *Bacteroides fragilis* 4-*O*-β-D-mannosyl-D-glucose phosphorylase; CE, cellobiose 2-epimerase; GH, glycoside hydrolase family; Glcβ1-4Man, β-D-glucopyranosyl-(1→4)-D-mannose; Manβ1-4Glc, β-D-mannopyranosyl-(1→4)-D-glucose; Manβ1-4Man, β-(1→4)-mannobiose; MGP, 4-*O*-β-D-mannosyl-D-glucose phosphorylase; MOP, β-1,4-mannooligosaccharide phosphorylase; Man1*P*, α-D-mannose 1-phosphate; *Ra*CE, *Ruminococcus albus* CE; *Ra*MP1, *R. albus* MGP; *Ra*MP2, *R. albus* MOP; *Rm*CE, *Rhodothermus marinus* CE; Uhgb_MP, β-1,4-mannopyranosyl-chitobiose phosphorylase from an uncultured gut

bacterium.

Carbohydrate isomerases/epimerases are essential in carbohydrate metabolism, and have great potential in industrial carbohydrate conversion. Cellobiose 2-epimerase (CE) reversibly epimerizes the reducing end D-glucose residue of β-(1→4)-linked disaccharides to D-mannose residue. CE shares catalytic machinery with monosaccharide isomerases and epimerases having an $(\alpha/\alpha)_6$-barrel catalytic domain. Two histidine residues act as general acid and base catalysts in the proton abstraction and addition mechanism. β-Mannoside hydrolase and 4-*O*-β-D-mannosyl-D-glucose phosphorylase (MGP) were found as neighboring genes of CE, meaning that CE is involved in β-mannan metabolism, where it epimerizes β-D-mannopyranosyl-(1→4)-D-mannose to β-D-mannopyranosyl-(1→4)-D-glucose for further phosphorolysis. MGPs form glycoside hydrolase family 130 (GH130) together with other β-mannoside phosphorylases and hydrolases. Structural analysis of GH130 enzymes revealed an unusual catalytic mechanism involving a proton relay and the molecular basis for substrate and reaction specificities. Epilactose, efficiently produced from lactose using CE, has superior physiological functions as a prebiotic oligosaccharide.

Key words: cellobiose 2-epimerase; 4-*O*-β-D-mannosyl-D-glucose phosphorylase; β-1,4-mannooligosaccharide phosphorylase; glycoside hydrolase family 130; epilactose

Carbohydrates are the most abundant chemical compounds in nature. Their structures including monosaccharide components, linkages, chain-lengths and modifications are diverse, and a wide variety of carbohydrate functions are known. They are the major energy source for organisms, and important for forming robust structures of plants, Crustacea, insects and microorganisms, resistance to abiotic stresses and intercellular communication. Several oligosaccharides are known to have beneficial physiological functions, such as prebiotic properties to improve intestinal microflora[1-4] and immune modulating activity.[5,6] A huge number of carbohydrate-metabolizing enzymes, such as glycoside hydrolases, glycosyltransferases, glycoside phosphorylases and sugar isomerases/epimerases, are involved in the formation and breakdown of carbohydrates. These enzymes are important not only for biological sugar metabolism but also for industrial carbohydrate conversion.

Carbohydrate isomerases and epimerases catalyze the epimerization (interconversion of epimers, EC 5.1.3.-) and isomerization (interconversion of aldose to ketose, EC 5.3.1-) of carbohydrates, respectively. These enzymes play very important roles in the metabolism of various carbohydrates, including in glycolysis, the oxidative/reductive pentose phosphate pathways and the Leloir pathway. Among these enzymes, D-xylose isomerase (EC 5.3.1.5), involved in D-xylose metabolism,[7,8] is particularly important in the food industry because it is used in the production of D-fructose-rich syrup.[9] Furthermore, this enzyme promotes the production of biofuel from lignocellulosic biomass.[10] D-Tagatose 3-epimerase (EC 5.1.3.31)[11] and D-psicose 3-epimerase (EC 5.1.3.30),[12] isolated from *Pseudomonas cichorii* and *Agrobacterium tumefaciens*, respectively, made possible the production of a rare sugar, D-psicose, from D-fructose.[14] D-Psicose is not used by humans,[13] and is expected to be used as a food material with a low glycemic index.[15] Furthermore, it exhibits antiobesity activity by increasing energy expenditure.[16,17]

Cellobiose 2-epimerase (EC 5.1.3.11; CE), which was first found in a ruminal bacterium *Ruminococcus albus*, catalyzes epimerization of a D-glucose residue at the

reducing end of cellobiose to a D-mannose residue, i.e. cellobiose is converted to β-D-glucopyranosyl-(1→4)-D-mannose (Glcβ1-4Man).[18] CE is the sole enzyme catalyzing the epimerization of oligosaccharides among tens of known carbohydrate isomerases/epimerases. Although CE was found by Tyler and Leatherwood about 50 years ago,[18] its structure, function in carbohydrate metabolism and application to functional oligosaccharides were only reported in the past decade. In this review, recent advances in the study of CE and structurally and functionally related enzymes are addressed.

I. Biochemical functions and distribution of CE

CE from *R. albus* (*Ra*CE) was successfully purified from cell-free extract of *R. albus* by column chromatography.[19] The molecular mass of *Ra*CE was estimated to be 43 kDa by SDS-PAGE analysis. As well as cellobiose, the enzyme had epimerization activity toward cellotriose, cellotetraose, lactose and β-(1→4)-mannobiose (Manβ1-4Man).[19-21] β-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-mannose, β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-mannose, epilactose [β-D-galactopyranosyl-(1→4)-D-mannose], and β-D-mannopyranosyl-(1→4)-D-glucose (Manβ1-4Glc) were generated from cellotriose, cellotetraose, lactose, and Manβ1-4Man, respectively. In the reactions with lactose and cellobiose, the conversion levels of epilactose and Glcβ1-4Man were both approximately 30%.[22,23] Among β-(1→4)-linked disaccharides, Manβ1-4Man is the best substrate of *Ra*CE in terms of catalytic efficiency (Table 1).[21] No epimerization activity of *Ra*CE was detectable toward monosaccharides (*N*-acetyl-D-glucosamine, uridine 5′-diphosphate-D-glucose, D-glucose 6-phosphate, D-glucose, and D-mannose) or glucobioses linked other than by a β-(1→4)-linkage [e.g. maltose: α-(1→4)-linkage; sophorose: β-(1→2)-linkage; laminaribiose: β-(1→3)-linkage; or gentiobiose: β-(1→6)-linkage].[20] Thus *Ra*CE is highly specific to a β-(1→4)-linkage at the reducing end of substrates, but can catalyze conversion of substrates with a D-glucosyl,

D-mannosyl or D-galactosyl residue next to the reducing end sugar residue.

Based on the deduced amino acid sequence of *Ra*CE, CE-like genes were found in various genome-sequenced bacteria including anaerobes and aerobes. CEs have been characterized using recombinant enzymes from *Bacteroides fragilis*,[24] *Caldicellulosiruptor saccharolyticus*,[25] *Cellulosilyticum lentocellum*,[26] *Cellvibrio vulgaris*,[27] *Dictyoglomus turgidum*,[28] *Dyadobacter fermentans*,[29] *Dysgonomonas gadei*,[26] *Eubacterium cellulosolvens*,[30] *Flavobacterium johnsoniae*,[29] *Herpetosiphon aurantiacus*,[29] *Pedobacter heparinus*,[29] *Rhodothermus marinus*,[23] *Saccharophagus degradans*,[29] *Spirosoma linguale*,[29] *Spirochaeta thermophila*,[31] and *Teredinibacter turnerae*[29] (Table 1). Seventy-one CE-like gene fragments were obtained from environmental DNA (rumen contents, soil, sugar beet extract and anaerobic sewage sludge) through a PCR-based metagenomics approach, and biochemical properties of two of them (mD1 and mD2) were investigated (Table 1).[32] These findings suggest that CE is distributed in various bacteria present in various environments.

All the characterized CEs have optimal pH around neutral, but their optimum temperatures are different depending on the growth temperature of the source organism of the enzyme. CEs from thermophiles such as *C. saccharolyticus*, *D. turgidum* and *R. marinus* are stable at a high temperature, and show their highest activity at 70–80°C.[23,25,28] Similar to *Ra*CE, these enzymes generally do not use disaccharide substrates other than β-(1→4)-linked disaccharides, although the CEs from *D. turgidum* and *S. thermophila* have measurable activity toward maltose and maltotriose.[28,31] Kinetic parameters for the epimerization of Manβ1-4Man have been examined with only a few CEs, but the k_{cat}/K_m values for this disaccharide are higher than those for cellobiose and lactose.[21,27,31,33] This fact is consistent with the prediction that CE is involved in the intracellular metabolism of β-(1→4)-mannooligosaccharides, as described later (see section III). Cellobiose was used as the substrate when CE was discovered, and this enzyme was named “cellobiose 2-epimerase”. However, considering its substrate selectivity and physiological function, it would be better to call

this epimerase "β-(1→4)-mannobiose 2-epimerase" (CE is used throughout this review according to the systematic name of Enzyme Nomenclature). Known CEs generally have higher k_{cat}/K_m for cellobiose than for lactose; only *R. marinus* CE (*Rm*CE) prefers lactose to cellobiose.[23] Epilactose, the epimerized product formed from lactose by CE, has beneficial physiological properties as described later (see section V), and thus *Rm*CE has an attractive substrate selectivity for use as an epilactose-producing enzyme.

Most known CEs catalyze only epimerization reactions; however, surprisingly, the CEs from *C. saccharolyticus* and *D. turgidum* also catalyze isomerization (producing epilactose and lactulose from lactose as the epimerization and isomerization products, respectively).[25,28] In the reactions of both these enzymes with lactose, conversion levels of epilactose and lactulose finally reached 13–15% and 55–58%, respectively.[34] This product distribution is notably different from that of other CEs that catalyze only epimerization.

In spite of high sequence identity with known CEs (32–42%), protein EpiA from *C. vulgaris* (also called *Cellvibrio mixtus*) has been considered to be an epimerase converting D-mannose to D-glucose.[35] However, biochemical analysis clearly demonstrated that this enzyme has a much higher preference for disaccharide substrates including Manβ1-4Man than D-mannose (k_{cat}/K_m for Manβ1-4Man is 5.5×10^4-fold higher than for D-mannose), indicating that *C. vulgaris* EpiA is a typical CE.[27] It is worth mentioning that the activity toward β-(1→4)-mannotriose is 0.24% of that toward Manβ1-4Man. This substrate selectivity is highly consistent with that of the CEs from *D. turgidum* and *S. thermophila*.[28,31] These findings suggest that CE has high disaccharide-selectivity, although epimerization activity toward oligosaccharides longer than a disaccharide was reported for several CEs.[19,24,29] Interestingly, *C. saccharolyticus* CE has low substrate selectivity. Its activities toward monosaccharide substrates, D-glucose, D-mannose, D-xylose, D-lyxose, and D-fructose, are 4.2, 1.1, 1.7, 1.0, and 0.26% of that toward cellobiose, respectively.[25]

II. Structure-function relationship of CE

Amein and Leatherwood reported that Glcβ1-4Man, produced from cellobiose by CE in D_2O, has deuterium at the C2 position of the reducing end D-mannose residue,[36] indicating that the CE reaction proceeds *via* a *cis*-enediol intermediate generated by the abstraction of 2H from the reducing end sugar moiety. Structural analysis of *Ra*CE and *Rm*CE revealed that both these CEs have an $(\alpha/\alpha)_6$-barrel-fold catalytic domain (Fig. 1A).[37,38] The catalytic domain and active site of CEs are similar to those of *N*-acylglucosamine 2-epimerases (EC 5.1.3.8; AGE)[39,40] and aldose-ketose isomerases (AKI; catalyzing interconversion of D-glucose, D-mannose and D-fructose)[41] with root mean square deviation values of 3.2 Å and 2.7 Å, respectively, although the overall amino acid sequence similarity between the CEs and these enzymes is low. Substrate and reaction specificities of CEs, AGEs and AKIs are very different, but their structural similarity indicates that they share common catalytic machinery and mechanism, especially in the formation of the *cis*-enediol intermediate.

Based on structural and mutational analysis of *Anabaena* sp. CH1 AGE, Lee *et al.* proposed a proton abstraction and donation mechanism involving two catalytic His residues (His239 and His372).[40] These His residues correspond to the His residues of pig kidney AGE (His248 and His382), predicted from the crystal structure to be essential for catalysis because they are close to an undefined substrate ligand.[39] His243 and His374 of *Ra*CE, corresponding to these two essential His residues of AGEs, were shown to be essential for catalytic activity by site-directed mutational analysis.[42] The structure of a complex of *Rm*CE and cellobiitol, which is an open-form substrate analog, resulted in a better understanding of the functions of the two catalytic His residues in the proton abstraction/donation mechanism (Fig. 1B).[38] The D-glucitol part of cellobiitol bound to the enzyme in a *cis*-enediol-like conformation in which the O2 atom of the D-glucitol part was rotated around the C2-C3 bond by about 90° from the equatorial position of the O2 atom in the ideal conformation of cellobiose. The C1-C2 bond of the D-glucitol part of cellobiitol was positioned between the two catalytic His

residues (His259 and His390). This structure was considered to be the state just before the abstraction of the H2 proton. His390 is in proximity to the H2 proton of the D-glucitol part of cellobiitol, suggesting that this residue is the most feasible candidate for abstracting the H2 proton in the reaction with the reducing end D-glucose residue (Fig. 1C). The other catalytic His residue, His259, is thought to act as a general acid catalyst, donating a proton to the *cis*-enediol intermediate. In the reverse reaction, His390 and His259 act as a general acid and base catalysts, respectively. Consistent with this observation, His248 of *Salmonella enterica* AKI (protein YihS), corresponding to His259 of *Rm*CE, is considered to be a general base catalyst in the reaction with D-mannose to generate the *cis*-enediol intermediate.[41] In AKI, His248 is predicted to give the proton, abstracted from H2 of D-mannose, to the C1 of the intermediate to form D-fructose. Considering this prediction, the His residues of *C. saccharolyticus* and *D. turgidum* CEs, corresponding to His259 of *Rm*CE, might transfer H2 to C1 of the *cis*-enediol intermediate to catalyze the isomerization reaction of the reducing end D-glucose or D-mannose residue. However, structural information on these CEs is not sufficient to conclusively determine the mechanism of isomerization catalyzed by CEs, and further analysis is required for better understanding.

Binding of cellobiitol to *Rm*CE indicated the requirement for ring opening, which is a common process as the first step of sugar isomerization/epimerization. In the ring opening, a general acid catalyst donates a proton to the endocyclic oxygen atom, whereas a general base catalyst abstracts a proton from the O1 atom (Fig. 1C). Based on the complexed structures of *Rm*CE and closed substrates (Glcβ1-4Man and epilactose),[38] His390 of *Rm*CE is considered to be the sole candidate for the general acid catalyst to donate a proton to O5 of the reducing end sugar residue (Fig. 1D). His200, Glu262 and His390 were suggested as candidates to be a general base catalyst abstracting a proton from O1 of the same residue. The Nε2 atom of His390 was close to both the O5 and O1 atoms of the reducing end of substrates, and thus this residue is predicted to be the most likely candidate for the general acid/base catalyst in the ring

opening. The mechanism of ring closure is interpreted as the reverse reaction of ring opening. After the epimerization of the reducing end sugar residue, rotation of the C2-C3 bond may occur again to bring the O1 atom of the reducing end sugar residue close to the O5 atom of the same residue.

In contrast to monosaccharide-specific AGE and AKI, CE is highly specific for disaccharides. Structural analysis of the enzyme-substrate complex of *Rm*CE[38] clearly identified important amino acid residues for the recognition of the non-reducing end of disaccharides (Fig. 1D). In the complexes of *Rm*CE with Glcβ1-4Man and epilactose, Trp385 on the α11→α12 loop of the enzyme stacks onto the non-reducing end glycosyl residue, and Ser185 and Asp188 on the α5→α6 loop form hydrogen bonds with 4OH and 6OH, respectively. Ser185 and Trp385 are completely conserved in CEs, and many CEs have Asp at the position of *Rm*CE Asp188. In pig AGE,[39] an aromatic amino acid residue, Phe377, is situated at the position corresponding to Trp385 of *Rm*CE, but the structure of the α5→α6 loop is very different to that in *Rm*CE and this loop could not interact with the non-reducing end sugar residue of a disaccharide substrate. AKI from *S. enterica* has α5→α6 and α11→α12 loops with distinct orientations from those in *Rm*CE, and no functionally equivalent amino acid residues are situated at the positions corresponding to Ser185, Asp188 and Trp385 of *Rm*CE.[41] Furthermore, superimposition of Glcβ1-4Man bound to *Rm*CE onto the structure of *S. enterica* AKI showed that the α7→α8 loop of *S. enterica* AKI clashes with the non-reducing end glucosyl residue, suggesting severe steric hindrance prevents disaccharide binding. These structural differences are presumably the reasons why AGE and AKI lack isomerization/epimerization activity toward disaccharide substrates.

AGE uses monosaccharide substrates harboring an *N*-acetyl group at the C2 position unlike the substrates of CE and AKI. Although the structure of the enzyme-substrate complex of AGE is still unknown, the structure of *Rm*CE complexed with substrates indicates the structural elements of AGE that might be responsible for binding to the acetamide group of its substrate (Fig. 1E). In the *Rm*CE-substrate

complexes, His200, Tyr124 and Asn196 form hydrogen bonds with the 2OH group of the reducing end sugar moiety. Although these amino acid residues are well conserved in AKIs, they are substituted by hydrophobic amino acid residues in AGEs. These three hydrophobic amino acid residues would form a small pocket, which is predicted to accommodate the acetamide group of the AGE substrate.

III. Physiological function of CE in carbohydrate metabolism

Genes neighboring the CE gene suggested the function of CE in carbohydrate metabolism. A putative endo-1,4-β-mannanase (EC 3.2.1.78) gene is encoded near the CE gene in the genome of most CE-producing bacteria for which data are available (Fig. 2A), suggesting that CE is involved in β-mannan metabolism. In *B. fragilis*, the CE gene (*BF0774*) is encoded downstream of the ManA gene (*BF0771*) belonging to glycoside hydrolase family (GH) 26. Cotranscription of *BF0771-BF0774* was confirmed,[33] and these genes constitute an operon. *BF0773* encodes a putative sugar/cation symporter. Biochemical analysis of recombinant protein encoded by *BF0772* demonstrated that it catalyzes the specific phosphorolysis of Manβ1-4Glc to α-D-mannose 1-phosphate (Man1*P*) and D-glucose;[33] this enzyme was named 4-*O*-β-D-mannosyl-D-glucose phosphorylase (EC 2.4.1.281; MGP). Product distribution analysis of ManA protein, predicted to be an extracellular enzyme, showed that this enzyme is a mannan 1,4-β-mannobiosidase (EC 3.2.1.100)[43] that specifically produces Manβ1-4Man from β-mannan and β-(1→4)-mannooligosaccharides. Based on these findings, CE is predicted to be involved in the following metabolic pathway (the CE-MGP pathway; Fig. 2B): Manβ1-4Man generated from β-mannan by extracellular ManA is imported into the cytosol through symporter BF0773. CE epimerizes Manβ1-4Man to Manβ1-4Glc, and MGP phosphorolyzes Manβ1-4Glc to Man1*P* and D-glucose, which are further metabolized through the Embden-Meyerhof-Parnas pathway. A gene organization similar to that in *B. fragilis* is present in the genome of *R. marinus* (Fig. 2A). MGP activity of a MGP-like protein, Rmar_2440, was confirmed

using a recombinant enzyme preparation.[44] The addition of glucomannan to the culture broth of *R. marinus* clearly induced the production of extracellular β-mannanase and the intracellular enzymes MGP and CE.[44] Bacterial growth was also enhanced by supplementation with glucomannan. This observation supports the idea that β-mannanase, CE and MGP are involved in the metabolism of β-mannan through the CE-MGP pathway.

In the genome of *R. albus*, the CE gene is not found in the gene cluster that was observed in *B. fragilis* and *R. marinus*, but two MGP-like genes (*Rumal_0099* and *Rumal_0852*) are present. The deduced amino acid sequences of *Rumal_0852* (*Ra*MP1) and *Rumal_0099* (*Ra*MP2) are 59% and 27% identical to that of *B. fragilis* MGP (*Bf*MGP), respectively. MGP activity was detected in cell-free extract of *R. albus*, and both MGPs were obtained from the cell-free extract, indicating that these two genes are both expressed in *R. albus*.[45] The detailed enzymatic properties of *Ra*MP1 and *Ra*MP2 were investigated using recombinant enzyme samples.[45] *Ra*MP1 is a typical MGP, having high selectivity for Manβ1-4Glc, but *Ra*MP2 is not, having markedly higher phosphorolytic activity toward β-(1→4)-mannooligosaccharides longer than Manβ1-4Man compared with its activity toward Manβ1-4Glc. Thus *Ra*MP2 was named β-1,4-mannooligosaccharide phosphorylase (MOP; EC 2.4.1.319). The phosphorolytic activity of *Ra*MP2 toward Manβ1-4Man is low (the k_{cat}/K_m is 22-fold lower than that for β-(1→4)-mannotriose), indicating that the physiological function of *Ra*MP2 is to generate Manβ1-4Man from longer β-(1→4)-mannooligosaccharides (Fig. 2B). Manβ1-4Man so generated would be metabolized by *Ra*MP1 and *Ra*CE through the CE-MGP pathway predicted in *B. fragilis*.

The gene cluster for the CE-MGP pathway in *C. vulgaris* includes three genes, *unkA*, *epiA* and *man5A*: *unkA* encodes a MGP, *epiA* encodes a CE and *man5A* encodes a β-mannosidase that liberates D-mannose from the non-reducing end of β-(1→4)-mannooligosaccharides (Fig. 2A). The α-galactosidase gene *aga27A* was also found downstream of *man5A*. Aga27A is considered to split the α-(1→6)-D-galactosyl

branch in galactomannan.[35] Man5A has much higher hydrolytic activity toward β-(1→4)-mannotriose and β-(1→4)-mannotetraose than Manβ1-4Man.[46] This substrate selectivity implies that this enzyme produces Manβ1-4Man for further metabolism by MGP and CE. The difference in enzymes for the degradation of β-(1→4)-mannooligosaccharides in *R. albus* and *C. vulgaris* might correlate with the efficiency of ATP production. Phosphorylation using ATP is required for the metabolism of free monosaccharides. In *R. albus*, MOP is used to avoid the generation of free D-mannose so as to save ATP.

IV. Functions and structures of GH130 mannoside phosphorylases

As described above, CE is considered to be involved in β-mannan metabolism along with MGP and MOP. According to sequence-based classification of carbohydrate active enzymes,[47] MGP and MOP are members of GH130 together with 1,4-β-mannosyl-*N*-acetylglucosamine phosphorylase (EC 2.4.1.320),[48] β-1,4-mannopyranosyl-chitobiose phosphorylase,[49] β-1,2-mannobiose phosphorylase,[50] 1,2-β-oligomannan phosphorylase[50] and β-1,2-mannosidase.[51,52] Phylogenetic analysis further classified GH130 members into subfamilies GH130_1, GH130_2 and GH130_NC (Fig. 3A): GH130_1 includes MGP; GH130_2 includes MOP, 1,4-β-mannosyl-*N*-acetylglucosamine phosphorylase and β-1,4-mannopyranosyl-chitobiose phosphorylase; and GH130_NC includes β-1,2-mannobiose phosphorylase, 1,2-β-oligomannan phosphorylase and β-1,2-mannosidase.[49] 1,4-β-Mannosyl-*N*-acetylglucosamine phosphorylase and β-1,4-mannopyranosyl-chitobiose phosphorylase, identified in gut *Bacteroides*, are considered to be involved in the degradation of *N*-glycan together with GH18 endo-β-*N*-acetylhexosaminidase (EC 3.2.1.96) and GH92 α-mannosidases (EC 3.2.1.24).[48,49] β-1,2-Mannobiose phosphorylase and 1,2-β-oligomannan phosphorylase were predicted to be involved in the biosynthesis of GDP-D-mannose, for which they supply Man1*P* for the reaction of mannose 1-phosphate guanylyltransferase (EC

2.7.7.22).[50] β-1,2-Mannosidase, identified in the intestinal bacterium *Bacteroides thetaiotaomicron*, is considered to remove the β-(1→2)-mannosyl cap of yeast α-mannan,[51] facilitating further degradation of the glycan by the concerted actions of various enzymes.[53] β-1,2-Mannosidase was also found in the aerobic bacterium *Dyadobacter fermentans*.[52]

Nakae *et al.* reported the three-dimensional structure of *Bf*MGP, the first of a GH130 member.[54] The catalytic domain of *Bf*MGP is made up of a five-bladed β-propeller fold (Fig. 4A). It has long α-helices at the N- and C-termini, and it forms a homohexamer through hydrophobic contacts *via* these α-helices. There is no acidic amino acid residue that could directly donate a proton as a general acid catalyst to the scissile glycosidic oxygen in the complex of *Bf*MGP with Manβ1-4Glc. The D-mannosyl residue bound to the −1 subsite adopts a stressed $B_{2,5}$ boat conformation (Fig. 4B). From these structural features, a unique reaction mechanism was postulated (Fig. 4C): the conserved Asp residue (Asp131 in *Bf*MGP) donates a proton to the 3OH of the D-mannosyl residue in the −1 subsite, and then the 3OH group gives a proton to the glycosidic oxygen to split the β-mannosidic linkage (i.e., there is a proton relay mechanism). This reaction mechanism is definitely different from that of the inverting phosphorylases categorized in GH families, because the general acid catalyst of such inverting phosphorylases directly donates a proton to the scissile glycosidic oxygen.[55-57] The 3OH group of the D-mannosyl residue in the −1 subsite is situated in an intermediate position between the catalytic Asp and the scissile glycosidic oxygen in other GH130 enzymes,[58,59] and these structural data support the proton relay reaction mechanism postulated by Nakae *et al.*,[54] although β-mannosidase is thought to transfer the proton from the general acid catalyst to the glycosidic oxygen *via* solvent.[51]

Structural analysis of *Ra*MP1 revealed that, unlike *Bf*MGP, this enzyme forms a homotrimer.[59] This difference in the quaternary structure is thought to be due to the substitution of hydrophobic amino acid residues found in the N-terminal α-helix of *Bf*MGP with hydrophilic residues in *Ra*MP1. Furthermore, in this structural analysis, it

was found that a loop, named Loop3, from the adjacent monomer contributed to the formation of the +1 subsite, and restricted the formation of further "+" subsites, which is an important factor in the disaccharide specificity of the enzyme. His245 on Loop3, which is conserved only in GH130_1 subfamily enzymes (Fig. 3B), forms a hydrogen bond with the 2OH of the D-glucose residue in the +1 subsite of the neighboring monomer *via* a water molecule (Fig. 4D), and it was shown to be important for catalytic activity through site-directed mutation. His245 is involved in a hydrogen bond network including this water, Manβ1-4Glc, inorganic phosphate and Lys251 binding to the inorganic phosphate. As the substitution of His245 with Ala significantly decreased the affinity for Man1*P* in the reverse phosphorolysis, His245 is also important for substrate binding in the −1 subsite. His245 might contribute to substrate binding to the −1 subsite by stabilizing the hydrogen bond network.

Compared with disaccharide-specific GH130_1 MGPs, GH130_2 enzymes have wide-open substrate-binding sites.[58,59] GH130_2 enzymes accept long-chain substrates with a degree of polymerization ≥3, and this open substrate-binding site is necessary to accommodate substrates longer than disaccharides. In contrast to GH130_1, subfamily GH130_2 contains mannoside phosphorylases with different substrate specificities. MOP strongly prefers a D-mannose moiety to a *N*-acetyl-D-glucosamine moiety at the +1 subsite, whereas 1,4-β-mannosyl-*N*-acetylglucosamine phosphorylase and β-1,4-mannopyranosyl-chitobiose phosphorylase have high selectivity for an *N*-acetyl-D-glucosamine moiety. Structural analysis of homohexameric Uhgb_MP, a β-1,4-mannopyranosyl-chitobiose phosphorylase from an uncultured gut bacterium, suggested that Met67 and Phe203 (situated on Loop3 of the adjacent molecule) side chains form a hydrophobic pocket interacting with the methyl group of the *N*-acetyl-D-glucosamine moiety in the +1 subsite (Fig. 4E).[58] *Ra*MP2, a MOP from *R. albus*, has Met75 at the position corresponding to Met67 of Uhgb_MP, but its Loop3 is shorter than that of Uhgb_MP, and it has no hydrophobic interaction with the acetamide group.[59] This structural feature might determine the substrate specificity of GH130_2

enzymes.

β-Mannosidases, catalyzing hydrolysis of a non-reducing end β-(1→2)-mannosidic linkage with net inversion, were recently identified among GH130_NC members.[51,52] They lack basic amino acid residues that are highly conserved in GH130 phosphorylases and responsible for positioning phosphate (Fig. 3B). Inverting glycosidases catalyze hydrolysis through a single displacement mechanism, in which a general acid catalyst donates a proton to the glycosidic oxygen, and a water molecule, activated by a general base catalyst, performs nucleophilic attack on the anomeric carbon. In these β-mannosidases, two Glu residues, conserved only in β-mannosidases (Fig. 3B), are predicted to coordinate a water molecule for the nucleophilic attack. Substitutions of both these Glu residues completely abolished the catalytic activity,[51,52] and thus they were considered to act as the general base catalyst.

V. Application of CE for production of functional oligosaccharides

Epilactose is present at a very low level in heated milk and alkaline-treated lactose.[60,61] Its physiological function was unclear for a long time because of difficulty in preparing this compound. Miyasato and Ajisaka reported enzymatic production of epilactose by transgalactosylation catalyzed by β-galactosidase.[62] In this reaction system, a D-galactosyl residue is transferred from a synthetic substrate, *p*-nitrophenyl β-D-galactopyranoside, to acceptor D-mannose. However, it is very difficult to synthesize epilactose on a >10-g scale by this method. Finding the epimerization activity of CE toward readily available lactose[20] made large-scale preparation of epilactose possible; in this reaction, approximately 30% of the lactose is converted to epilactose.[22,23] A continuous reaction system using CE immobilized on anion exchange resin effectively reduces the amount of enzyme required for epilactose synthesis.[63] The epilactose generated can be purified from the reaction mixture in several steps to approximately 90% purity; these steps include crystallization of residual lactose, hydrolysis of lactose by β-galactosidase, degradation of the resulting monosaccharides

by yeast and column chromatography using Na-form cation exchange resin.[22] All these steps can be scaled up, thus this purification procedure can be used industrially.

In vitro experiments revealed that epilactose is very stable with respect to intestinal digestive enzymes and enhances the growth of some bifidobacterial strains.[20] Stimulation of growth of bifidobacteria was confirmed in animal experiments using rats.[64] Oral administration of epilactose inhibited conversion of primary bile acids to secondary bile acids,[64] which are considered to be risk factors for colon cancer, and stimulated mineral absorption.[65] Enhanced mineral absorption is predicted to be caused by lowering the pH through the production of short chain fatty acids by intestinal bacteria proliferated by epilactose. Furthermore, Suzuki *et al*. clearly demonstrated that epilactose increased paracellular Ca^{2+} absorption in the small intestine by inducing the phosphorylation of myosin regulatory light chain by myosin light chain kinase and Rho-associated kinase.[66] As a result of enhanced mineral absorption following the ingestion of epilactose, postgastrectomy osteopenia and anemia were improved in rat experiments,[67] suggesting that epilactose is a superior candidate functional foodstuff to prevent osteoporosis and anemia. Furthermore, recently, we reported that epilactose effectively prevents high-fat diet-induced obesity in mice.[68] Epilactose feeding increased the expression of uncoupling protein 1, which is involved in dissipation of energy, in skeletal muscles and brown adipose tissue, leading to an increase in whole-body energy expenditure. The expression of uncoupling protein 1 is enhanced by propionic acid, which is generated by intestinal microflora that use epilactose as a carbon source. Thus epilactose has great potential to prevent obesity and related diseases, recognized as major health problems, particularly in developed countries.

The isomerization activity of CE from *C. saccharolyticus* is used in the efficient enzymatic production of lactulose,[34] a commercially available prebiotic oligosaccharide. Although epilactose is also generated as a byproduct (15% yield), a 58% yield of lactulose was obtained in the reaction of 700 g/L lactose substrate. As lactulose is currently produced by alkaline isomerization of lactose, the production process includes

complicated purification and desalting steps. An environmentally friendly synthetic method using a biocatalyst is preferable to the conventional chemical reaction.

CEs are fully active in milk, and thus direct production of epilactose and lactulose has been established.[26,69,70] Highly purified lactose is required for production of galactooligosaccharide and lactulose by chemical reaction, because the substrate concentration must be high enough for efficient transgalactosylation for galactooligosaccharide production and the purity of the substrate must be high for chemical isomerization of lactose to avoid side reactions. Thus, direct production of prebiotic oligosaccharides using CEs in milk is a very attractive alternative approach to easily convert milk to functional dairy products.

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Disclosure statement

There is no potential conflict of interest in this work.

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Figure legends

Fig. 1 Structure-function relationship of CE. A, Overall structure of *Rm*CE (PDB entry 3WKG). The numbering of α-helices is indicated. B, Comparison of orientations of Manβ1-4Glc (left; PDB entry 3WKG) and cellobiitol (right; PDB entry 3WKI) bound to the catalytic site of *Rm*CE. C, Proposed catalytic mechanism of epimerization of cellobiose. From the structural analysis, AH, which donates a proton to O5 in the ring opening, is predicted to be His390, and His200, Glu262 and His390 are candidates to be B^-, which abstracts a proton from O1. D, Enzyme-substrate interaction in *Rm*CE (PDB entry 3WKG). Amino acid residues surrounding Glcβ1-4Man bound to the active site are shown. Amino acid residues of *Rm*CE and Glcβ1-4Man are shown, respectively, in green and yellow stick representations. Yellow dashed lines indicate predicted hydrogen bonds. E, Comparison of reducing end sugar binding between *Rm*CE (left; PDB entry 3WKG) and AGE (right; PDB entry 1FP3). The reducing end D-mannose residue of Glcβ1-4Man bound to *Rm*CE is shown in the left-hand image. In the right-hand image, this D-mannose residue is superimposed into the pig AGE structure using PyMOL program v0.99rc6 (DeLano Scientific LLC, South San Francisco, CA, USA). Tyr124, Asn196 and His200 of *Rm*CE shown in green stick representation respectively correspond to Phe122, Ala180 and Met184 of pig AGE shown in magenta stick representation.

Fig. 2. The CE-MGP pathway for β-mannan metabolism. A, Organization of the gene cluster including the CE gene. Red, glycoside hydrolases splitting the β-(1→4)-mannosidic linkage of β-mannan and β-(1→4)-mannooligosaccharide; green, MGP; blue, putative symporters predicted to be involved in the uptake of Manβ1-4Man; orange, CE; and magenta, MOP. The genes involved in the CE-MGP pathway of *R. albus* do not constitute the gene cluster unlike the other bacterial strains shown here. B, Predicted metabolic pathway of β-mannan. MOP and β-mannosidase successively degrade β-(1→4)-mannooligosaccharide to Manβ1-4Man in *R. albus* and *C. vulgaris*,

respectively, whereas Manβ1-4Man is directly produced by ManA in *B. fragilis*. Degradation mechanism of β-(1→4)-mannooligosaccharide to Manβ1-4Man in *R. marinus* is unclear. Manβ1-4Man is epimerized to Manβ1-4Glc by CE, and the resulting Manβ1-4Glc is phosphorolyzed by MGP.

Fig. 3. Phylogenetic tree and multiple-sequence alignment of GH130 enzymes. A, Phylogenetic tree of characterized GH130 enzymes was constructed by the neighbor joining method using the ClustalW program (http://www.genome.jp/tools/clustalw/). B, Multiple-sequence alignment of GH130 enzymes. The names of subfamilies are shown on the left of the figure. Amino acid residues involved in phosphate binding are indicated by black circles. “B” below the sequence indicates general base catalyst residues of β-mannosidase. Loop 3 sequence is surrounded by a dashed line.

Fig. 4. Structure-function relationship of GH130_1 and GH130_2 mannoside phosphorylases. A, Overall structure of *Bf*MGP (PDB entry 3WAS). B, Manβ1-4Glc bound to the active site of *Bf*MGP (PDB entry 3WAS). Yellow dashed lines indicate predicted hydrogen bonds. C, catalytic mechanism of MGP. D, Hydrogen-bonding network found in *Ra*MP1 (PDB entry 5AYC). (A) and (B) show MolA and MolB respectively. Lys251, inorganic phosphate and Manβ1-4Glc in MolA (green) form a hydrogen bond network together with His245 from the adjacent molecule (MolB, blue). E, Comparison of structures of Loop3 between *Ra*MP2 (left) and Uhgb_MP (right). D-Mannose and *N*-acetyl-D-glucosamine bound to subsites −1 and +1 of Uhgb_MP (PDB entry 4UDK), respectively, were superimposed onto *Ra*MP2 (PDB entry 5AYE) using PyMOL program v0.99rc6. These sugars are bound to MolA (green). Loop3 from MolB (mainly in blue) is shown in orange.

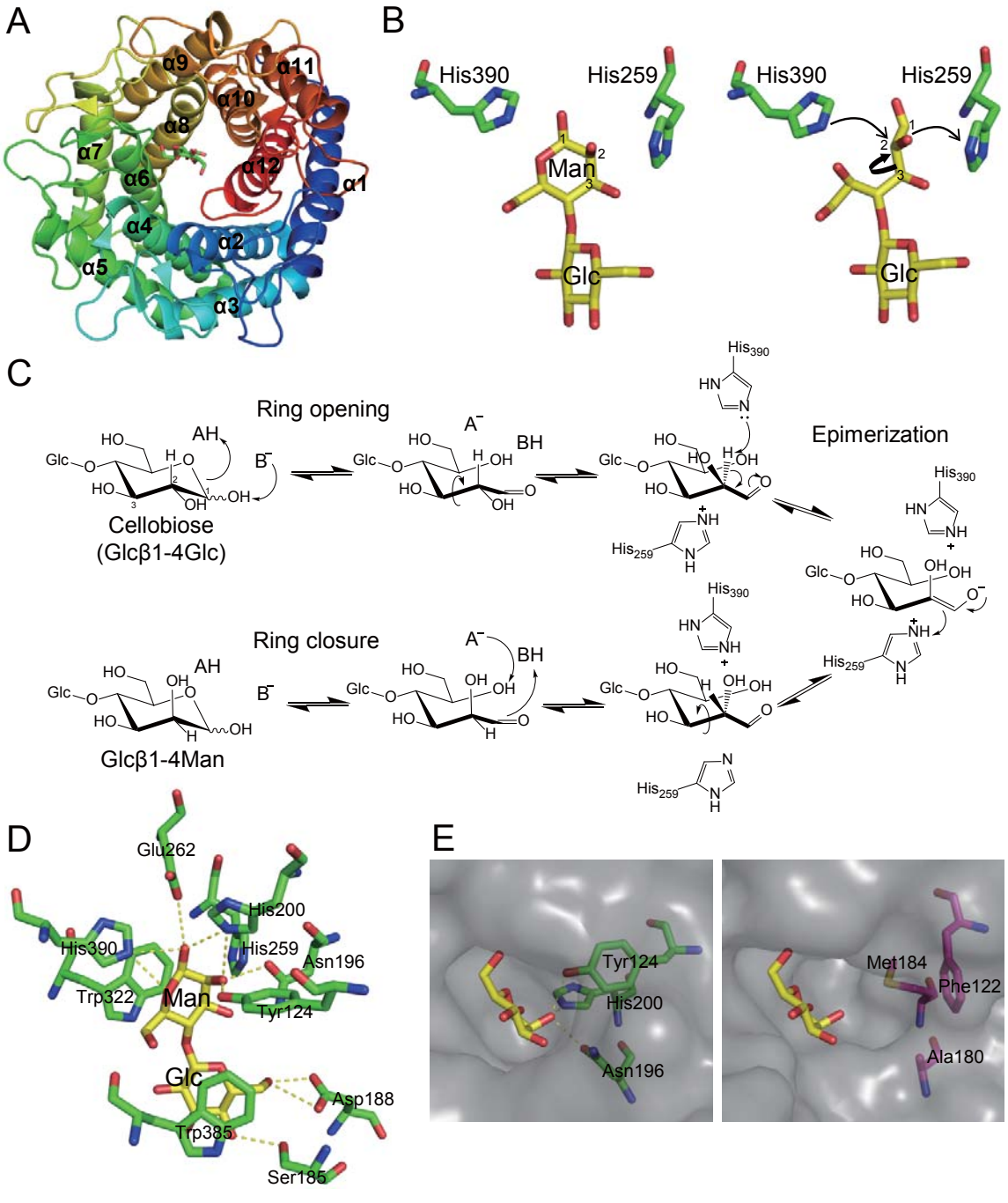

A
α9
α11
α10
α8
α7
α12
α1
α6
α4
α2
α5
α3
B
His390
His259
Man
Glc
His390
His259
Glc
C
Ring opening
Epimerization
Cellobiose
(Glcβ1-4Glc)
Ring closure
Glcβ1-4Man
D
Glu262
His200
His390
His259
Asn196
Trp322
Man
Tyr124
Glc
Asp188
Trp385
Ser185
E
Tyr124
His200
Asn196
Met184
Phe122
Ala180

Fig. 1, Sabri

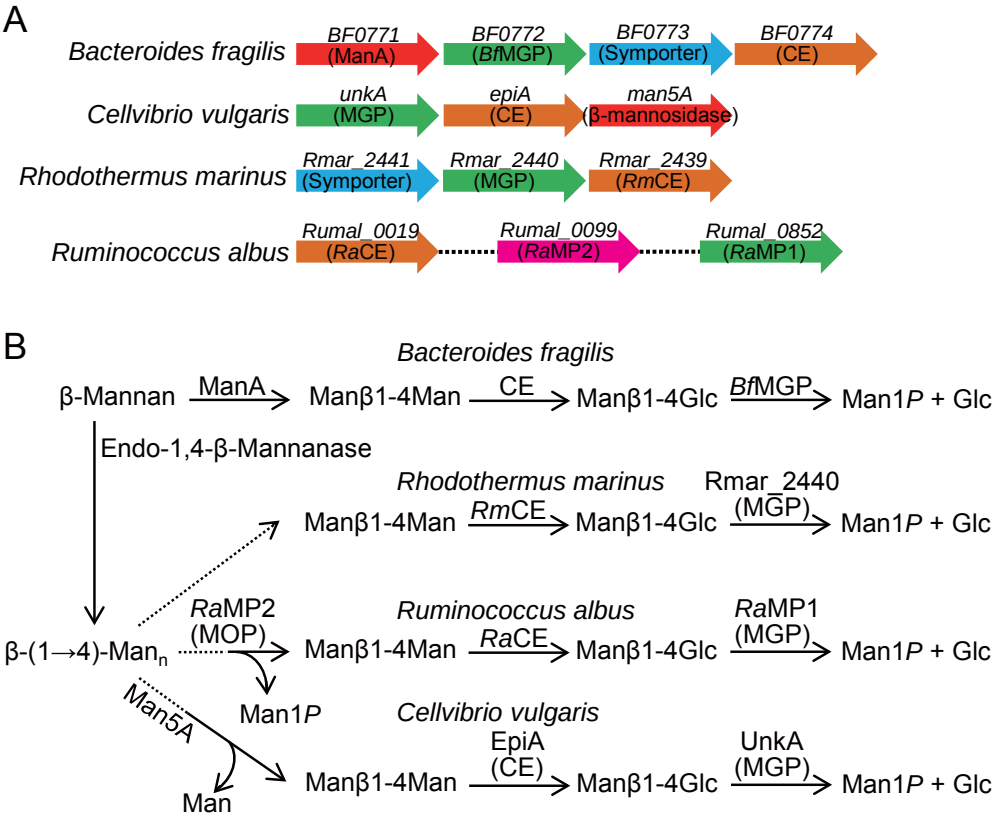
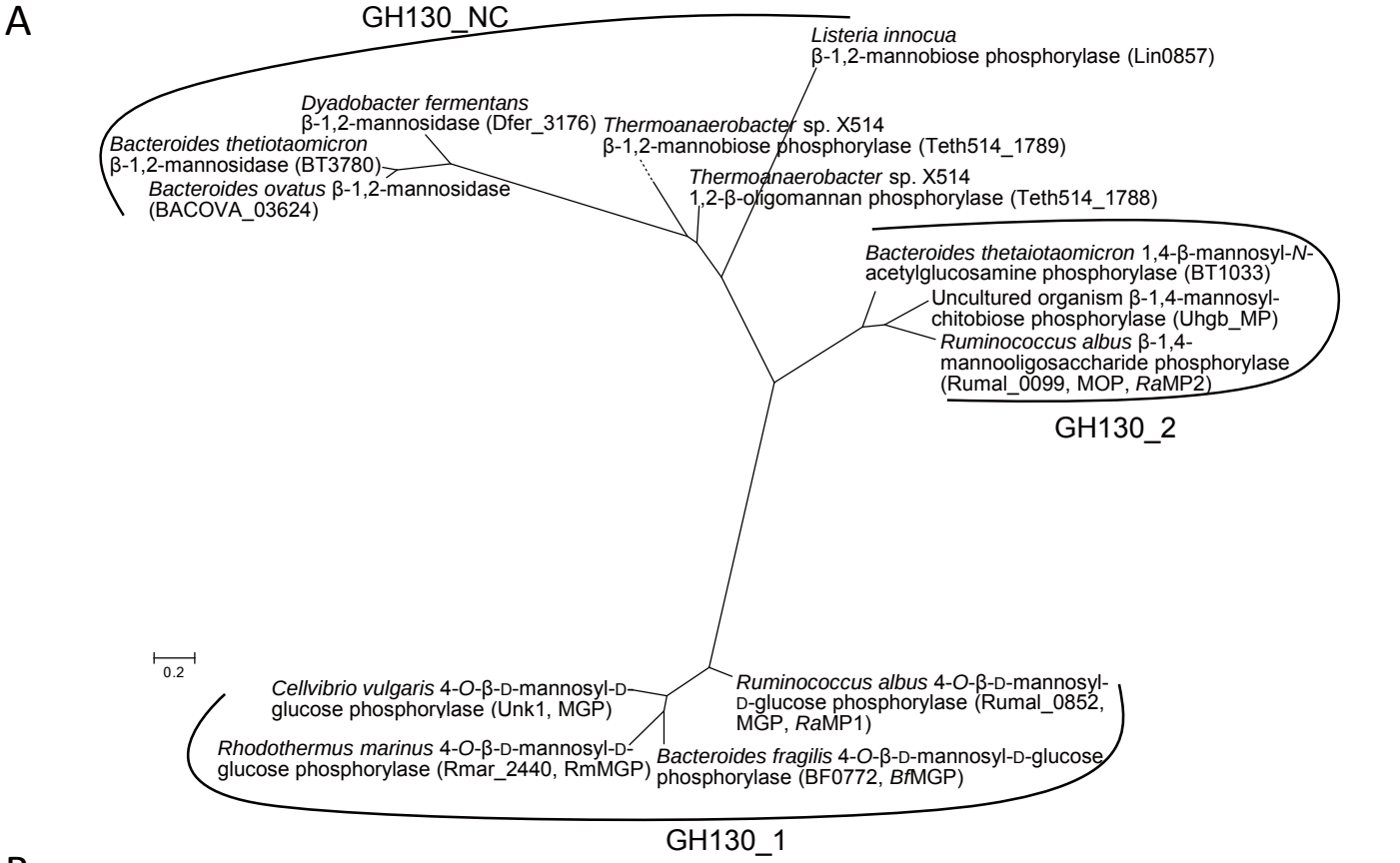

A
Bacteroides fragilis
BF0771 (ManA)
BF0772 (BfMGP)
BF0773 (Symporter)
BF0774 (CE)
Cellvibrio vulgaris
unkA (MGP)
epiA (CE)
man5A (β-mannosidase)
Rhodothermus marinus
Rmar_2441 (Symporter)
Rmar_2440 (MGP)
Rmar_2439 (RmCE)
Ruminococcus albus
Rumal_0019 (RaCE)
Rumal_0099 (RaMP2)
Rumal_0852 (RaMP1)
B
Bacteroides fragilis
β-Mannan
ManA
Manβ1-4Man
CE
Manβ1-4Glc
BfMGP
Man1P + Glc
Endo-1,4-β-Mannanase
Rhodothermus marinus
Rmar_2440 (MGP)
RmCE
Manβ1-4Man
Manβ1-4Glc
Man1P + Glc
β-(1→4)-Man$_n$
RaMP2 (MOP)
Man1P
Ruminococcus albus
RaMP1 (MGP)
RaCE
Manβ1-4Man
Manβ1-4Glc
Man1P + Glc
Man5A
Man
Cellvibrio vulgaris
EpiA (CE)
UnkA (MGP)
Manβ1-4Man
Manβ1-4Glc
Man1P + Glc

Fig. 2, Saburi



B

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GH130_                    260        280        300        320
  1 BfMGP      : TAGIARTKDLKNWERLPDLKT-KS----------QQRNVVLHP---------EFVDGKYALYTRPQDGFIDTGSGGGIGW : 225
    RaMP1      : SAGIARTKDLTNWERLPDLVTLRSP---------QQRNVTLLP---------EFVDGKYAFYTRPMDGFIETGSGGGIGF : 222
    RmMP1      : QCGIARTKDLKTWERLPDLKT-PSP---------QQRNCVLHP---------EFVDGKYAFYTRPQDDFIEAGSGGGIGW : 233
    Unk1       : QCGIARTKDLITWERLPDLVT-YSG---------QQRNVVLHP---------EFIDGKYGLFTRPQDGFISVGAGGGIGW : 225
  2 RaMP2      : TIGVAYTFDFKTFYQCENAFL---P---------FNRNGVLFP---------RKINGKYVMFSRPSDSGHT--PFGDMFI : 194
    Uhgb_MP    : TIGVAYTFDFETFHQLENAFI---P---------FNRNGVLFP---------RKINGRFAMLSRPSDNGHT--PFGDIFY : 182
    BT1033     : TIGIAYTFDFVDFFQCENAFL---P---------FNRNGVLFP---------QKIDGKYAMLSRPSDNGHT--PFGDIYI : 179
 NC Teth514_1788 : RICLATSKNLIDWERKGVVLD---E---------PNKDASLFP---------EKINGKYVMLHR---------RYPDIWI : 167
    Teth514_1789 : RICMVWSDDLKNWKGHRIVLD---E---------PNKDAALLS---------EKINGKYVLFHR---------RMPDIWI : 173
    Lin0857    : ADALVTTKDFENLEYQGNIFA---P---------ENKDVLIFP---------EKINGKYYALHRPSLKSI---GNLDIWI : 219
    BT3780     : RLAIATSRNLKDWTKHGPAFA---KAYDGKFFNLGCKSGSILTEVVNGKQVIKKIDGKYFMYWGE----------EHVFA : 232
    BACOVA_03624 : RLAVATSRNLKDWTKHGPAFA---KAFDGKFFNLGCKSGSILTEVVKGKQVIKKVNGKYFMYWGE----------EHVFA : 235
    Dfer_3176  : RLGVATSRDLLKWDKQGPAFA---KAYGGKFNNIWTKSGSILTKLVGDKQVIAKVNGKYWLYFGE----------ANVNI : 229
                                                                                  B

GH130_                    340        360        380        400
                                  Loop3
  1 BfMGP      : ALIDDITHAEV-----GEEKILDKRYYH---TIKEVKNGEGPHPIKTPQGWLHLAHGVRNCAA-------GLRYVLYMYM : 290
    RaMP1      : GLADDITHAVI-----DEERMTSIRRYH---TITESKNGAGATPIKTERGWLNIAHGVRNTAA-------GLRYVIYCFV : 287
    RmMP1      : GLCADITNPVI-----TEERIVDPRVYH---TIKEAKNGLGPPPIKTPHGWLHLAHGVRNTAA-------GLRYVLYLFM : 298
    Unk1       : GLVDDMTRAEV-----KSEVIVDGKVYH---TIKEVKNGQGPAPIKTAEGWLNLAHGVRNTAA-------GLRYVLYMFM : 290
  2 RaMP2      : SQSPDMKYWG-------EHRHVMGP----LRAWESKKIGAGPIPIETSEGWLCFYHGVLESCN-------GFVYSFSACI : 256
    Uhgb_MP    : SESPDMEFWG-------RHRHVMSPAAFEVSAWQCTKIGAGPIPVETPEGWLLIYHGVLHSCN-------GYVYSFGSAL : 248
    BT1033     : SYSPDMKYWG-------EHRCVMKVTPFPESAWQCTKIGAGSVPFLTDEGWLLFYHGVITTCN-------GFRYAMGSAI : 245
 NC Teth514_1788 : AFSDDLKNWY-------DHKPILKPIP---NTWESARVGIGGPPIKIKDGWFLIYHAADD----------NNVYRLGAVL : 227
    Teth514_1789 : AYSDDLVNWY-------NHKIIMSPKS---HTWESKKIGIAGPPIKREDGWLLIYHGVDN----------NNVYRLGVAL : 233
    Lin0857    : ASSPDLRSFG-------DHRHLLGIRP---GEYDSGRVGGGCVPIKTEEGWLILYHGATE----------ENRYVMGAAL : 279
    BT3780     : ATSEDLVNWTPYVNTDGSLRKLFSPRD---GHFDSQLTECGPPAIYTPKGIVLLYNGKNSASR-GDKRYTANVYAAGQAL : 308
    BACOVA_03624 : ATSDDLIHWTPIVNIDGSLKKLFSPRD---GYFDSHLTECGPPAIYTPKGIVLLYNGKNHSGR-GDKRYTANVYAAGQAL : 311
    Dfer_3176  : ATSTDLLNWEPVVDSKGDLINLISPRK---GYFDSNLTECGPPAILTDKGIVLLYNGKNDKGEDGDKRFTPNSYCAGQVL : 306
                                                                                  B
```

Fig. 3, Saburi

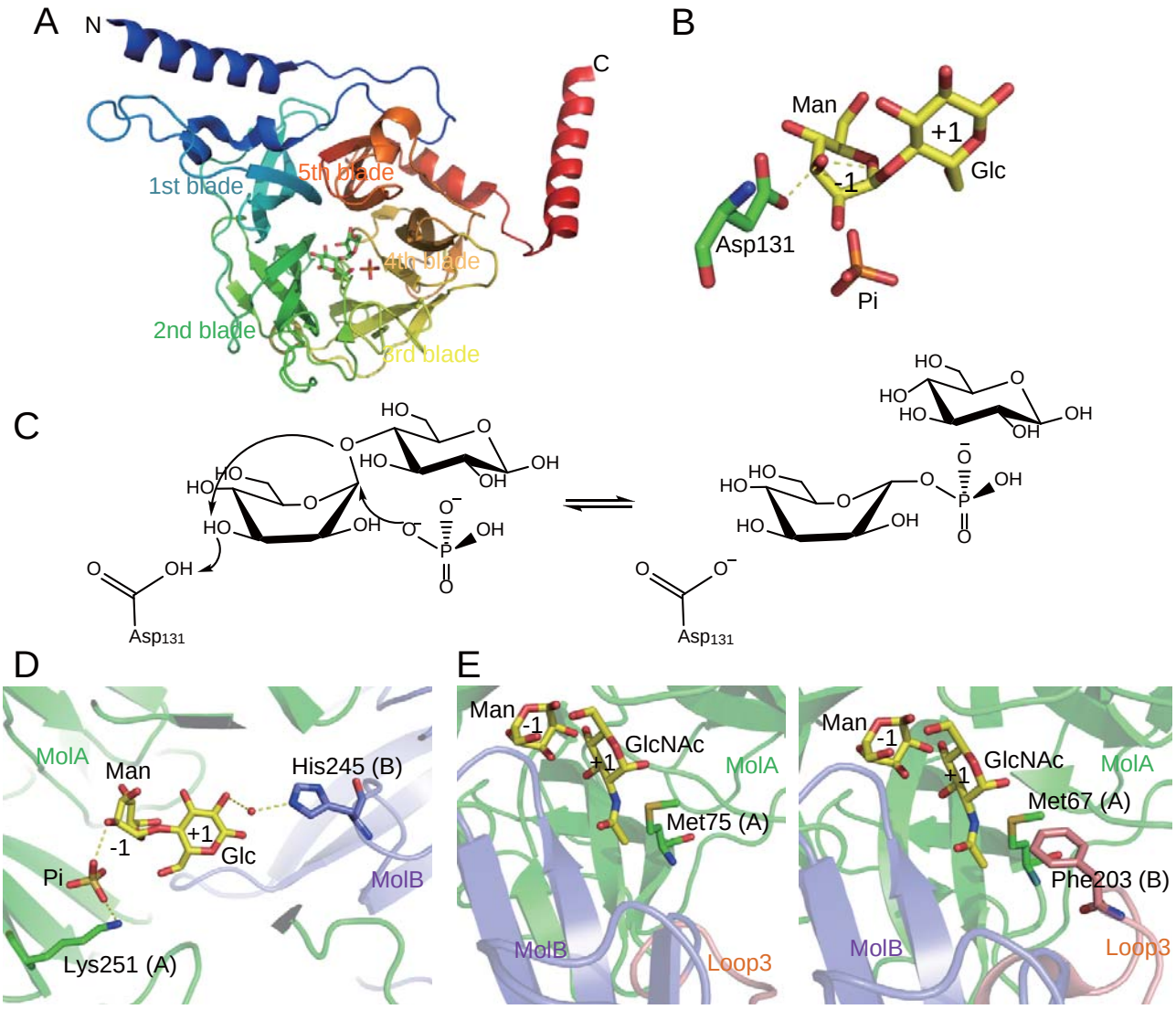
A
N
C
1st blade
5th blade
4th blade
2nd blade
3rd blade
B
Man
+1
-1
Glc
Asp131
Pi
C
HO
O
OH
Asp131
D
MolA
Man
His245 (B)
+1
-1
Glc
Pi
MolB
Lys251 (A)
E
Man
-1
GlcNAc
+1
MolA
Met75 (A)
MolB
Loop3
Man
-1
GlcNAc
+1
MolA
Met67 (A)
Phe203 (B)
MolB
Loop3

Fig. 4. Saburi

Table 1. Biochemical properties of CEs from various bacterial origins.

Bacterial strain	pH		Temperature		Cellobiose			Lactose			β-(1→4)-Mannobiose			Reference
	Optimum	Stability[a]	Optimum (°C)	Stability (°C)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	
Bacteroides fragilis	7.5	N.D.	45	N.D.	67.6[f]	3.75[f]	18.0[f]	79.5[f]	6.56[f]	12.1[f]	104[f]	5.51[f]	18.9[f]	24
Caldicellulosiruptor saccharolyticus	7.5	N.D.	75	≤80[c]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	25
Cellvibrio vulgaris	8.0	5.2-10.0	40	≤40[c]	38.8[g]	24.5[g]	1.58[g]	18.6[g]	28.7[g]	0.648[g]	102[g]	4.03[g]	25.3[g]	27
Dictyoglomus turgidum	7.0	N.D.	70	≤80[c]	169[i]	75.8[i]	2.23[i]	89.4[i]	79.6[i]	1.12[i]	N.D.	N.D.	N.D.	28
Dyadobacter fermentans	7.7	3.2-10.2	50	≤50[b]	240[f]	179[f]	1.34[f]	44.9[f]	95.7[f]	0.469[f]	N.D.	N.D.	N.D.	29
Eubacterium cellulosolvens	7.5	4.0-8.0	35	≤40[d]	28.5[f]	11.3[f]	2.52[f]	32.5[f]	72.0[f]	0.451[f]	N.D.	N.D.	N.D.	30
Flavobacterium johnsoniae	8.4	4.7-9.8	35	≤30[b]	39.9[f]	53.2[f]	0.750[f]	17.5[f]	34.9[f]	0.501[f]	N.D.	N.D.	N.D.	29
Herpetosiphon aurantiacus	7.3	8.0-9.4	45	≤50[b]	18.7[f]	28.2[f]	0.663[f]	14.0[f]	51.9[f]	0.270[f]	N.D.	N.D.	N.D.	29
Pedobacter heparinus	6.3	5.3-11.8	35	≤30[b]	7.02[f]	29.6[f]	0.237[f]	5.43[f]	24.5[f]	0.222[f]	N.D.	N.D.	N.D.	29
Rhodothermus marinus	6.3	3.2-10.8	80	≤80[c]	80.8[i]	27.2[i]	2.97[i]	111[i]	28.8[i]	3.85[i]	102[i]	5.25[i]	19.4[i]	23
Ruminococcus albus	7.5	N.D.	30	N.D.	63.8[f]	13.8[f]	4.62[f]	52.1[f]	33.0[f]	1.58[f]	201[g]	8.74[g]	23.0[g]	20, 21
Saccharophagus degradans	7.7	4.7-10.8	35	≤30[b]	26.1[f]	22.6[f]	1.15[f]	7.82[f]	29.2[f]	0.268[f]	N.D.	N.D.	N.D.	29
Spirosoma linguale	7.7	2.2-9.5	45	≤50[b]	222[f]	104[f]	2.13[f]	92.1[f]	206[f]	0.447[f]	N.D.	N.D.	N.D.	29
Spirochaeta thermophila	7.0	N.D.	60	≤60[b]	28.0[h]	4.30[h]	6.51[h]	N.D.	N.D.	N.D.	35.8[h]	3.50[h]	10.2[h]	31
Teredinibacter turnerae	8.8	3.4-10.2	35	≤45[b]	165[f]	198[f]	0.833[f]	175[f]	238[f]	0.735[f]	N.D.	N.D.	N.D.	29
Rumen content environmental DNA (mD1)	N.D.	N.D.	N.D.	N.D.	113[e]	25.1[e]	4.50[e]	N.D.	N.D.	N.D.	166[e]	3.92[e]	42.8[e]	32
Rumen content environmental DNA (mD2)	N.D.	N.D.	N.D.	N.D.	137[f]	21.8[f]	6.33[f]	N.D.	N.D.	N.D.	109[f]	10.8[f]	11.0[f]	32

Stable range of pH was evaluated from residual activity after incubation at various pHs at 4°C for 24 h (a). Thermal stability was evaluated from residual activity after incubation at various temperature for 15 min (b), 30 min (c), or 60 min (d). Kinetic parameters were determined at 25°C (e), 30°C (f), 37°C (g), 60°C (h), and 70°C (i). N.D., not determined.