Running title: Cellobiose 2-Epimerase from Aerobes

Identification and Characterization of Cellobiose 2-Epimerases from Various Aerobes

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Abbreviations: CE, cellobiose 2- epimerase; AGE, *N*-acyl-D-glucosamine 2-epimerase; RaCE, CE from *Ruminococcus albus* NE1; BfCE, CE from *Bacteroides fragilis* NCTC 9343; EcCE, CE from *Eubacterium cellulosolvens* NE13; CsCE, CE from *Caldicellulosiruptor saccharolyticus*; DtCE, CE from *Dictyoglomus turgidum*; RmCE, CE from *Rhodothermus marinus* JCM 9785; NCBI, the National Center for Biotechnology Information; TLC, thin layer chromatography; FjCE, CE from *Flavobacterium johnsoniae* NBRC 14942; ESI-MS, electron spray ionization mass spectrometry; NMR, nuclear magnetic resonance; SdCE, CE from *Saccharophagus degradans* ATCC 43961; SlCE, CE from *Spilosoma linguale* ATCC 33905; HaCE, CE from *Herpetosiphon aurantiacus* ATCC 23779; TtCE, CE from *Teredinibacter turnerae* ATCC 39867; DfCE, CE from *Dyadobacter fermentans* ATCC700827; PhCE, CE from *Pedobacter heparinus* NBRC 12017

Cellobiose 2-epimerase (CE), found mainly in anaerobes, reversibly converts D-glucose residues at the reducing end of β-1,4-linked oligosaccharides to D-mannose residues. In this study, we characterized CE-like proteins from various aerobes (*Flavobacterium johnsoniae* NBRC 14942, *Pedobacter heparinus* NBRC 12017, *Dyadobacter fermentans* ATCC 700827, *Herpetosiphon aurantiacus* ATCC 23779, *Saccharophagus degradans* ATCC 43961, *Spirosoma linguale* ATCC 33905, and *Teredinibacter turnerae* ATCC 39867), because aerobes, more easily cultured on a large scale than anaerobes, are applicable in industrial processes. The recombinant CE-like proteins produced in *Escherichia coli* catalyzed epimerization at the C2 position of cellobiose, lactose, epilactose, and β-1,4-mannobiose, whereas *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, D-glucose, and D-mannose were inert as substrates. All the CEs, except for *P. heparinus* CE, the optimum pH of which was 6.3, showed highest activity at weakly alkaline pH. CEs from *D. fermentans*, *H. aurantiacus*, and *S. linguale* showed higher optimum temperatures and thermostability than the other enzymes analyzed. The enzymes from *D. fermentans*, *S. linguale*, and *T. turnerae* showed significantly high k_{cat} and K_m values towards cellobiose and lactose. Especially, *T. turnerae* CE showed a very high k_{cat} value towards lactose, an attractive property for the industrial production of epilactose, which is carried out at high substrate concentrations.

Key words: cellobiose 2-epimerase; *N*-acetyl-D-glucosamine 2-epimerase; substrate specificity; epilactose; epimerization

Cellobiose 2-epimerase (CE; EC 5.1.3.11), found in *Ruminococcus albus*,[1] reversibly catalyzes 2-epimerization at the reducing end of β-1,4-linked oligosaccharides such as cellobiose and lactose. It shows low sequence similarity to *N*-acyl-D-glucosamine 2-epimerases (AGEs), and catalyzes the interconversion of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosamine. Particular residues of both classes of enzymes are commonly essential for catalytic function.[2]

Since the *R. albus* NE1 CE (RaCE) gene was cloned,[3] CE genes from certain anaerobes, *Bacteroides fragilis* NCTC9343 (BfCE), *Eubacterium cellulosolvens* NE13 (EcCE), *Caldicellulosiruptor saccharolyticus* (CsCE), and *Dictyoglomus turgidum* (DtCE), have been identified.[4-7] The BfCE gene is found in an operon along with the genes encoding β-mannanase and 4-*O*-β-D-mannosyl-D-glucose phosphorylase, which specifically phosphorolyzes 4-*O*-β-D-mannosyl-D-glucose to α-D-mannosyl phosphate and D-glucose,[8] suggesting that the physiological role of CE is to convert β-1,4-mannobiose to Man-Glc for further phosphorolysis. Recently we identified CE from aerobic *Rhodothermus marinus* JCM 9785 (RmCE),[9] and hence this enzyme is not distributed only in anaerobes but also in aerobes.

CE is an attractive enzyme for the production of functional food stuffs. Epilactose (4-*O*-β-D-galactosyl-D-mannose), produced from lactose with CE, has a prebiotic property, proliferating lactobacilli and bifidobacteria in the gut.[10] Oral administration of this oligosaccharide inhibits the conversion of primary bile acids to secondary bile acids, which are suggested to promote colon cancer. Furthermore, it has been found that epilactose increases the intestinal absorption of minerals, including Ca and Fe, in rats.[11,12] Absorption of Ca in the rat small intestine was enhanced through activation of the paracellular route, which is mediated by Rho-associated kinase and myosin light chain kinase, by epilactose.[13] CsCE and DtCE have been found to catalyze not only epimerization at the C2-position but also isomerization, although the reaction velocity for isomerization is slower than for epimerization.[6,7] In the reaction of CsCE towards lactose, this enzyme produces lactulose, which also has prebiotic properties and is

utilized as an ingredient.[14] The final conversion ratios of lactose to epilactose and lactulose have been reported to be 15% and 60%, respectively. Recently, it was found that DtCE catalyzes the epimerization and isomerization of maltooligosaccharides in addition to epimerizations of cellooligosaccharides and lactose, although activity towards maltose is 70- and 40-fold lower than towards cellobiose and lactose, respectively.[7] The amino acid sequences of CsCE and DtCE are 38-45% identical those of the other known CEs (RaCE, BfCE, EcCE, and RmCE). This sequence identity is as high as the identity among the later four enzymes (35-46%), and the structural elements important for substrate specificity and isomerization activity are not easily identified based on sequence comparison of CEs.

For the industrial application of bacterial enzymes, enzymes from aerobes are useful, because aerobes can be more easily cultured in large scale than anaerobes. In this study, we identified and characterized CEs in several aerobes, *Flavobacterium johnsoniae* NBRC 14942, *Pedobacter heparinus* NBRC 12017, *Dyadobacter fermentans* ATCC 700827, *Herpetosiphon aurantiacus* ATCC 23779, *Saccharophagus degradans* ATCC 43961, *Spirosoma linguale* ATCC 33905, and *Teredinibacter turnerae* ATCC 39867, to screen a new CE with favorable properties for industrial use.

Materials and Methods

Materials. D-Glucose, D-galactose, lactose, epilactose, cellobiose, sophorose, and D-mannose were purchased from Sigma (St. Louis, MO). Cellotriose and laminaribiose were from Seikagaku (Tokyo). D-Fructose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-mannosamine were from Nacalai Tesque (Kyoto, Japan). β-1,4-Mannobiose, gentiobiose, and maltose were from Megazyme (Wicklow, Ireland), Wako Pure Chemical Industries (Osaka, Japan), and Nihon Shokuhin Kako (Tokyo), respectively.

Bacterial strains. F. johnsoniae NBRC 14942 and *P. heparinus* NBRC 12017 were

purchased from the National Institute of Technology and Evaluation Biological Resource Center (Chiba, Japan). *D. fermentans* ATCC 700827, *H. aurantiacus* ATCC 23779, *S. degradans* ATCC 43961, *S. linguale* ATCC 33905, and *T. turnerae* ATCC 39867 were from the American Type Culture Collection (Manassas, VA). All the bacteria were cultured aerobically following the suppliers' instructions. *Escherichia coli* DH5α and *E. coli* BL21 (DE3)-Codonplus-RIL (Stratagene, La Jolla, CA) were used in the preparation of expression plasmids and the production of recombinant proteins, respectively.

Construction of expression plasmids. The following CE-like genes of the indicated bacteria were obtained by PCR: *S. degradans*, NCBI reference sequence YP_525984; *S. linguale*, YP_003385134; *H. aurantiacus*, YP_001543663; *P. heparinus*, YP_003094236; *F. johnsoniae*, YP_001197274; *T. turnerae*, YP_003075376; and *D. fermentans*, YP_003086363. The chromosomal DNA of each bacterium, prepared as described previously,[9] was used as template. PCR was performed using KOD Plus DNA polymerase (Toyobo, Osaka, Japan). The primers harboring the required restriction sites are listed in Table 1. The *Nde*I sites in the putative CE genes of *H. aurantiacus* and *T. turnerae* were deleted by the introduction of silent mutations by the overlapping PCR method.[15] All target genes other than that of *T. turnerae* were cloned into the *Nde*I and *Xho*I sites of the pET22b vector (Novagen, Darmstadt, Germany). The target gene of *T. turnerae* was cloned using the same vector and the *Nde*I and *Eco*RI sites. The sequences of the cloned genes were analyzed with CEQ 2000XL (Beckman Coulter, Brea, CA). Thirty-four nucleotides in the CE-like gene of *T. turnerae* (nine amino acids in the deduced amino acid sequence) were different from the registered sequence, although the DNA sequences of the other genes were entirely identical to them. The strain of *T. turnerae* used in this study was the same as the strain the genome sequence of which was analyzed, and hence some errors might have occurred in the sequence analysis of this bacterium. The DNA sequence of the CE-like

gene of *T. turnerae* was deposited in the DNA Data Bank of Japan under accession no. AB719057.

Table 1

Production and purification of the recombinant CE-like proteins. The transformant of *E. coli* BL21 (DE3)-Codonplus-RIL harboring a given expression plasmid was cultured in 0.5 L of Luria-Bertani medium containing 100 μg/mL of ampicillin at 30°C until A_{600} reached 0.5. One-hundred millimolar isopropyl 1-thio-β-D-galactoside (Wako Pure Chemical Industries) was added to a final concentration of 0.1 mM, and cell culturing was continued at 16°C for 24 h. Bacterial cells, harvested by centrifugation, were suspended in 20 mL of 20 mM sodium phosphate buffer (pH 7.0, buffer A) and disrupted by sonication. The cell-free extract obtained by centrifugation was applied to a DEAE Toyopearl 650M column (ϕ1.6 × 15 cm, Tosoh, Tokyo) equilibrated with buffer A. The adsorbed protein was eluted by a linear gradient of 0–0.5 M NaCl (total elution volume, 120 mL). The active fractions were pooled and dialyzed against 5 mM sodium phosphate buffer (pH 7.0, buffer B). The resulting solution was applied to a hydroxyapatite Bio-Gel HT column (ϕ1.6 × 10 cm, Bio-Rad, Hercules, CA) equilibrated with buffer B. The adsorbed protein was eluted by a linear gradient of sodium phosphate buffer (pH 7.0) of 5–200 mM (total elution volume, 60 mL). The purity of the fractions was analyzed by SDS-PAGE. Active fractions were pooled and concentrated to several mg/mL by ultrafiltration using Amicon Ultra-15 concentrators (Millipore, Billerica, MA). The protein concentration was determined by the Bradford method,[16] in which bovine serum albumin was used as standard. An equal volume of glycerol was added to the purified samples for storage at -20°C.

Substrate specificity and structural analysis of the reaction products. A reaction mixture of 20 μL, consisting of 10 μg/mL of enzymes, 100 mM substrates, and buffer A, was incubated at 30°C for 24 h. D-Glucose, D-galactose, lactose, epilactose, cellobiose, sophorose, D-mannose, cellotriose, laminaribiose, β-1,4-mannobiose, gentiobiose,

maltose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-mannosamine were tested as substrates. One microliter of the mixture was analyzed by thin-layer chromatography (TLC; Silica gel 60 F_{254}, Merck, Darmstadt, Germany) using a solvent system of 1-butanol/2-propanol/water (2/2/1, v/v/v). The chromatogram was visualized by spraying with 10% v/v sulfuric acid in methanol and heating the TLC plate.

The reaction products from lactose, epilactose, cellobiose, and β-1,4-mannobiose were characterized. A reaction mixture of 0.7 mL, consisting of 100 mg/mL of substrate, 100 μg/mL of enzyme, and buffer A, was incubated at 30°C for 24 h. The reaction was terminated by boiling for 3 min, and the products were purified by HPLC, as described previously.[9] The chemical structures of the oligosaccharides produced by the *F. johnsoniae* CE-like protein (FjCE) were determined by electron spray ionization mass spectrometry (ESI-MS) and ^{13}C nuclear magnetic resonance (NMR). ESI-MS was done using an LCT Premier XE (Waters, Milford, MA), in which the positive ion was analyzed. ^{13}C NMR spectra were recorded at 25°C in D_2O with an Inova500 (125 MHz, Agilent, Santa Clara, CA). The peak arising from dioxane at 67.40 ppm was standardized. The structures of the oligosaccharides produced by the other enzymes were confirmed by monosaccharide analysis. A mixture of 50 μL consisting of 2 mg/mL of the product and 2 M trifluoroacetic acid was kept at 100°C for 3 h. The material was dried *in vacuo*, and the residue was dissolved in 20 μL of water. A one microliter aliquot was analyzed by TLC as described above. Carbohydrate was detected using a detection reagent consisting of ethanol/sulfuric acid/acetic acid/anisaldehyde (92/3/2/3, v/v/v/v).

Enzyme characterization. A one milliliter of reaction mixture consisting of an appropriate concentration of the enzyme, 100 mM lactose, and buffer A was incubated at 30°C for 10 min. The enzymatic reaction was terminated by adding 0.2 mL of 0.1 *N* HCl and boiling the sample for 3 min. As internal standard, 0.3 mL of 5 mg/mL sorbitol was added, and the epilactose produced was measured by HPLC, as described

previously.[9] The optimum pH and temperature were determined by measuring the activity at various pH values (pH 3–13) and temperatures (25–55°C), respectively. Britton-Robinson buffer (pH 3–13, composed of an acidic mixture of 20 mM acetic acid, 20 mM phosphoric acid, and 20 mM glycine, the pH value of which was adjusted with NaOH) was used as the reaction buffer to vary the reaction pH. Temperature and pH stabilities were evaluated by measuring residual activity after the temperature and pH treatments, respectively. As temperature treatment, the enzyme was incubated at 25–55°C for 15 min. As pH treatment, it was kept in 10 mM Britton-Robinson buffer at 4°C for 24 h. Ranges in which the enzyme retained more than 90% of the original activity were considered stable. The kinetic parameters for the epimerization of lactose and cellobiose were determined by measuring the initial reaction rates toward 10–350 mM of the substrates and fitting the results to the Michaelis-Menten equation.

Phylogenetic analysis. The amino acid sequences of CEs and related enzymes including putative proteins (172 sequences) were collected by a sequence similarity search of RaCE with the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and a phylogenetic tree was constructed by the Neighbor-Joining method[17] with ClustalW.[18] The graphic was illustrated by MEGA 5.05.[19]

Results and Discussion

Selection and preparation of target proteins

A sequence similarity search of RaCE was carried out with the Basic Local Alignment Search Tool, and a phylogenetic tree was constructed (Fig. 1). Known CEs fell into a cluster distant from the cluster of AGEs. CsCE and DtCE, which catalyze the isomerization of lactose and act on maltooligosaccharide, respectively,[7,14] showed close relationships with RaCE and EcCE. Seven aerobes harboring CE-like proteins, *F.*

johnsoniae NBRC 14942, *P. heparinus* NBRC 12017, *D. fermentans* ATCC700827, *H. aurantiacus* ATCC23779, *S. degradans* ATCC43961, *S. linguale* ATCC33905, and *T. turnerae* ATCC39867, were selected to investigate the biochemical properties of the CE-like proteins. The amino acid sequences of these CE-like proteins were 35-39% identical to that of RaCE (Table 2). Fig. 1

The recombinant CE-like proteins produced in *E. coli* were purified to homogeneity by DEAE Toyopearl 650M and hydroxyapatite Bio-Gel HT column chromatography. The production levels of the recombinant enzymes in 0.5 L of the culture medium, estimated by the specific activity (towards 100 mM lactose) of the purified enzymes, were 13.8-176 mg (Table 2). The amounts of purified enzymes obtained were 5.11–84.0 mg. Table 2

Substrate specificity of the target proteins

All the recombinant proteins acted on cellobiose, lactose, epilactose, and β-1,4-mannobiose, whereas *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, D-glucose, and D-mannose did not serve as substrates. The enzymes from *S. degradans* (SdCE), *S. linguale* (SlCE), *H. aurantiacus* (HaCE), *T. turnerae* (TtCE), and *D. fermentans* (DfCE) also acted on cellotriose. All the enzymes produced a sole product in the reaction towards each substrate under the analytical conditions unlike CsCE and DtCE.[6,7)]

Structural analysis of the reaction products

The structures of the oligosaccharides produced from lactose, epilactose, cellobiose, and β-1,4-mannobiose by FjCE were determined by ESI-MS and ^{13}C-NMR. All of the products gave a signal at 365.1 *m/z* $[M + Na]^+$, corresponding to disaccharides. The chemical shifts in the ^{13}C-NMR analysis of the products from epilactose, cellobiose, and β-1,4-mannobiose were coincident with values that have been reported for lactose, 4-*O*-β-D-glucosyl-D-mannose, and 4-*O*-β-D-mannosyl-D-glucose, respectively,[20)] and

the chemical shifts of the product from lactose were identical to the chemical shifts of authentic epilactose. These results clearly indicate that FjCE catalyzes the 2-epimerization of D-glucose or D-mannose residues at the reducing ends of the substrates. The structures of the oligosaccharides produced by the other CE-like proteins were confirmed by analysis of the monosaccharide components, because all the oligosaccharides showed similar mobility to the corresponding oligosaccharides produced by FjCE. The reaction products derived from cellobiose, lactose, epilactose, and β-1,4-mannobiose were found to be composed of D-glucose and D-mannose, D-galactose and D-mannose, D-galactose and D-glucose, and D-glucose and D-mannose, respectively, indicating that all the CE-like proteins examined were also CEs. All the aerobes examined in this study have the putative 4-*O*-β-D-mannosyl-D-glucose phosphorylase genes. Hence the metabolic pathway of β-1,4-mannobiose postulated for an anaerobe, *B. fragilis*,[8] might be also distributed in aerobes.

Effects of pH and temperature on enzyme activity and stability

The effects of pH and temperature on the activity and stability of the CEs were investigated (Table 3). All the CEs, except for *P. heparinus* CE (PhCE), showed highest activity at weakly alkaline pH values, as observed for most known CEs. In contrast, the optimum pH of activity for PhCE was 6.3, as observed for RmCE. All the CEs showed high stability at alkaline pH, and DfCE, SlCE, and TtCE were more stable at acidic pH compared with the other enzymes. DfCE, HaCE, and SlCE showed higher optimum temperatures and thermostability than the other enzymes analyzed here.

Kinetic parameters for the epimerization of cellobiose and lactose

Table 3

DfCE, HaCE, SdCE, and SlCE showed 2.5–4.8-fold higher k_{cat}/K_m to cellobiose than to lactose similar to RaCE, BfCE, and EcCE. In contrast, the k_{cat}/K_m values of FjCE, PhCE, and TtCE towards both substrates were similar to RmCE (Table 4). DfCE, SlCE, and TtCE showed significantly high k_{cat} and K_m values. In particular, TtCE

showed the highest k_{cat} value towards lactose among the CEs analyzed in this study and reported thus far. The K_m value of TtCE for lactose was significantly high, but this should cause no problem in the application of the enzyme in epilactose production, because the industrial reaction is usually carried out at very high substrate concentrations (generally more than 0.5 M). In the reaction at a concentration higher than the K_m value, enzyme amount (mg) can be reduced if the enzyme has a high k_{cat} for the substrate. Therefore, TtCE is thought to be a candidate enzyme for the industrial production of epilactose.

Table 4

Acknowledgments

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References

1) Tyler TR and Leatherwood JM, *Arch. Biochem. Biophys.*, **119**, 383-387 (1967).
2) Ito S, Hamada S, Ito H, Matsui H, Ozawa T, Taguchi H, and Ito S, *Biotechnol. Lett.*, **31**, 1065-1071 (2009).
3) Ito S, Hamada S, Yamaguchi K, Umene S, Ito H, Matsui H, Ozawa T, Taguchi H, Watanabe J, Wasaki J, and Ito S, *Biochem. Biophys. Res. Commun.*, **360**, 640-645 (2007).
4) Senoura T, Taguchi H, Ito S, Hamada S, Matsui H, Fukiya S, Yokota A, Watanabe J, Wasaki J, and Ito S, *Biocsi. Biotechnol. Biochem.*, **73**, 400-406 (2009).
5) Taguchi H, Senoura T, Hamada S, Matsui H, Kobayashi Y, Watanabe J, Wasaki J, and Ito S, *FEMS Microbiol. Lett.*, **287**, 34-40 (2008).
6) Park CS, Kim JE, Choi JG, and Oh DK, *Appl. Microbiol. Biotechnol.*, **92**, 1187-1196 (2011).

7) Kim JE, Kim YS, Kang LW, and Oh DK, *Biotechnol. Lett.*, in press.
8) Senoura T, Ito S, Taguchi H, Higa M, Hamada S, Matsui H, Ozawa T, Jin S, Watanabe J, and Ito S, *Biochem. Biophys. Res. Commun.*, **408**, 701-706 (2011).
9) Ojima T, Saburi W, Sato H, Yamamoto T, Mori H, and Matsui H, *Biosci. Biotechnol. Biochem.*, **75**, 2162-2168 (2011).
10) Watanabe J, Nishimukai M, Taguchi H, Senoura T, Hamada S, Matsui H, Yamamoto T, Wasaki J, Hara H, and Ito S, *J. Dairy Sci.*, **91**, 4518-4526 (2008).
11) Nishimukai M, Watanabe J, Taguchi H, Senoura T, Hamada S, Matsui H, Yamamoto T, Wasaki J, Hara H, and Ito S, *J. Agric. Food Chem.*, **56**, 10340-10345 (2008).
12) Suzuki T, Nishimukai M, Shinoki A, Taguchi H, Fukiya S, Yokota A, Saburi W, Yamamot T, Hara H, and Matsui H, *J. Agric. Food Chem.*, **58**, 10787-10792 (2010).
13) Suzuki T, Nishimukai M, Takechi M, Taguchi H, Hamada S, Yokota A, Ito S, Hara H, and Matsui H, *J. Agric. Food Chem.*, **58**, 1927-1932 (2010).
14) Kim YS and Oh DK, *Bioresour. Technol.*, **104**, 668-672 (2012).
15) Ho SN, Hunt HD, Horton RM, Pullen JK, and Pease LR, *Gene*, **77**, 51-59 (1989).
16) Bradford MM, *Anal. Biochem.*, **72**, 248-254 (1976).
17) Saitou N and Nei M, *Mol. Biol. Evol.*, **4**, 406-425 (1987).
18) Thompson JD, Higgins DG, and Gibson TJ, *Nucleic Acids Res.*, **22**, 4673-4680 (1994).
19) Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S, *Mol. Biol. Evol.*, **28**, 2731-2739 (2011).
20) Bock K, Pedersen C, and Pedersen H, *Adv. Carbohydr. Chem. Biochem.*, **42**, 193-225 (1984).
21) Ito S, Taguchi H, Hamada S, kawauchi S, Ito H, Senoura T, Watanabe J, Nishimukai M, Ito S, and Matsui H, *Appl. Microbiol. Biotechnol.*, **79**, 433-441 (2008).

Figure legend

Fig. 1. Phylogenetic Tree of CEs and Related Enzymes.

The accession numbers of the Genbank or NCBI Reference Sequence are shown.

aAGE and pAGE are AGEs from *Anabaena* sp. CH1 and pig, respectively.

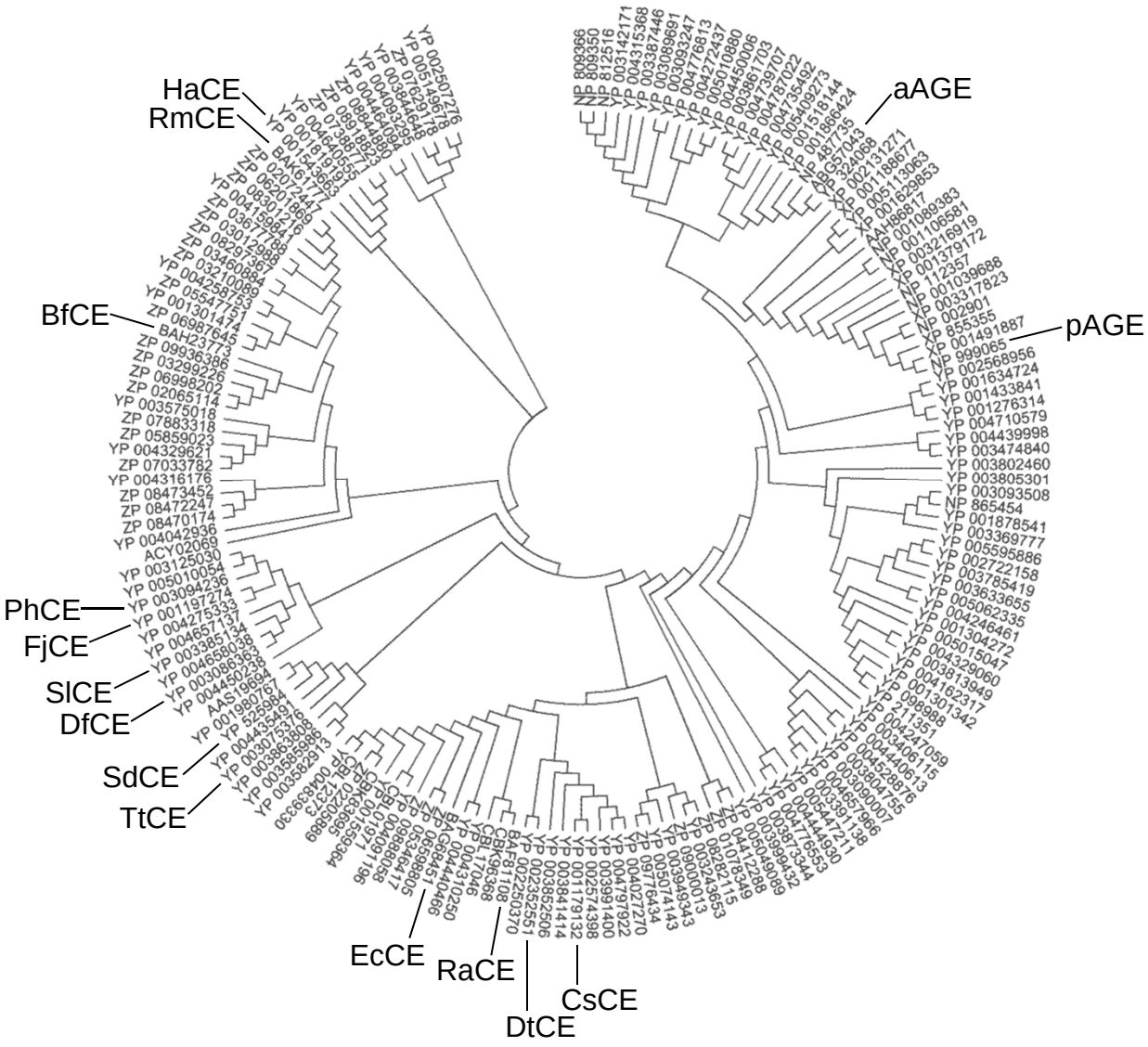

Fig. 1, Ojima et al.

Table 1. Sequences of the Primers Used in This Study

Name	Sequence (5'→3')
DfCE-F-NdeI	AAACATATGGCCGCATTTACACCCGAAG
DfCE-R-XhoI	GAACTCGAGTTACGCTTTGCCGGTCCTG
FjCE-F-NdeI	AAACATATGCCAGCAAATCTAAAACAGCTT
FjCE-R-XhoI	AAACTCGAGTTAGGTTTTAATTCGGTTAATAA
HaCE-F1-NdeI	AAACATATGGCGAATCTCGTAGATGG
HaCE-R1-del	GCCTCCATCATGTGCAACATGG
HaCE-F2-del	ACCATGTTGCACATGATGGAG
HaCE-R2-XhoI	AAACTCGAGTTACCCTCGGATCTGAACACTC
PhCE-F-NdeI	AAACATATGAGCGAAATTCTTATACAAGA
PhCE-R-XhoI	AAACTCGAGTCAGGAAAAATCGAAATTGACAC
SdCE-F-NdeI	AAGGGCATATGGGTGTGCCGCTTAAAAAAA
SdCE-R-XhoI	GGGCTCGAGTTATTCGTTATTACCAAATATTTCTC
SlCE-F-NdeI	AAGGGCATATGGACTTAAAGCAACTTCGCG
SlCE-R-XhoI	GGGCTCGAGTTATCCATGCAGATGATCCAGTCG
TtCE-F1-NdeI	AAACCCATATGGAAACTGAAACGGATAC
TtCE-R1-del	TTCATAAACATGTGAACGTGCC
TtCE-F2-del	ACGTTCACATGTTTATGAATAC
TtCE-R2-EcoRI	AAAGAATTCCTAACAGTCGACATTCAACAC

All primers were used in the preparation of the expression plasmids. The target enzymes are indicated in the names of the primers.

Table 2. Sequence Identity, Production Levels, and Yields of the CEs from Aerobes

Name		Identity (%)	Production Level (mg)	Yield (mg)
EcCE	*Eubacterium cellulosolvens* NE13	47	N.D.	N.D.
RmCE	*Rhodothermus marinus* JCM 9785	38	N.D.	N.D.
BfCE	*Bacteroides fragilis* NCTC 9343	39	N.D.	N.D.
CsCE	*Caldicellulosiruptor saccharolyticus* DSM 8903	44	N.D.	N.D.
DtCE	*Dictyoglomus turgidum* DSM 9724	43	N.D.	N.D.
DfCE	*Dyadobacter fermentans* ATCC 700827	36	84.2	84.0
FjCE	*Flavobacterium johnsoniae* NBRC 14942	35	176	75.9
HaCE	*Herpetosiphon aurantiacus* ATCC 23779	37	121	35.0
PhCE	*Pedobacter heparinus* NBRC 12017	39	43.1	7.31
SdCE	*Saccharophagus degradans* ATCC 43961	36	13.8	5.11
SlCE	*Spirosoma linguale* ATCC 33905	35	35.9	31.0
TtCE	*Teredinibacter turnerae* ATCC 39867	36	126	22.6

N.D., not determined. Sequence identity to RaCE is shown. The production level is the amount of enzymes produced in 0.5 L of culture broth.

Table 3. Effects of pH and Temperature on CEs from Various Aerobes

	pH		Temperature (°C)	
Enzyme	Optimum	Stability	Optimum	Stability
DfCE	7.7	3.2-10.2	50	≤50
FjCE	8.4	4.7-9.8	35	≤30
HaCE	7.3	8.0-9.4	45	≤50
PhCE	6.3	5.3-11.8	35	≤30
SdCE	7.7	4.7-10.8	35	≤30
SlCE	7.7	2.2-9.5	45	≤50
TtCE	8.8	3.4-10.2	35	≤45

Table 4. Kinetic Parameters for the Epimerization of Cellobiose and Lactose

Enzyme	Cellobiose			Lactose		
	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})
DfCE	240 ± 39.8	179 ± 32	1.34	44.9 ± 4.9	95.7 ± 23.9	0.469
FjCE	39.9 ± 3.23	53.2 ± 7.3	0.75	17.5 ± 0.5	34.9 ± 4.3	0.501
HaCE	18.7 ± 0.45	28.2 ± 5.3	0.663	14.0 ± 1.8	51.9 ± 6.8	0.27
PhCE	7.02 ± 0.21	29.6 ± 5.8	0.237	5.43 ± 0.06	24.5 ± 0.9	0.222
SdCE	26.1 ± 1.83	22.6 ± 4.4	1.15	7.82 ± 0.42	29.2 ± 4.4	0.268
SlCE	222 ± 2.89	104 ± 9.4	2.13	92.1 ± 12.2	206 ± 40	0.447
TtCE	165 ± 6.56	198 ± 13	0.833	175 ± 31	238 ± 45	0.735
RaCE[21)]	63.8	13.8	4.62	52.1	33.0	1.58
BfCE[4)]	67.6	3.75	18.0	79.5	6.56	12.1
EcCE[5)]	28.5	11.3	2.52	32.5	72.0	0.451
RmCE[9)]	80.8	27.2	2.97	111	28.8	3.85

Mean ± standard deviation for three independent experiments is shown. The kinetic parameters of the CEs except for RmCE were determined at 30°C, and the kinetic parameters of RmCE were measured at 60°C.