Two Novel Glycoside Hydrolases Responsible for the Catabolism of Cyclobis-(1→6)-α-nigerosyl*

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The actinobacterium Kribbella flavida NBRC 14399T produces cyclobis-(1→6)-α-nigerosyl (CNN), a cyclic glucotetraose with alternate α-(1→6)- and α-(1→3)-glucosidic linkages, from starch in the culture medium. We identified gene clusters associated with the production and intracellular catabolism of CNN in the K. flavida genome. One cluster encodes α-glucosyltransferase and 3-α-isomaltosyltransferase, which are known to coproduce CNN from starch. The other cluster contains four genes annotated as a transcriptional regulator, sugar transporter, glycoside hydrolase family (GH) 31 protein (Kfla1895), and GH15 protein (Kfla1896). Kfla1895 hydrolyzed the α-(1→3)-glucosidic linkages of CNN and produced isomaltose via a possible linear tetrasaccharide. The initial rate of hydrolysis of CNN (11.6 s⁻¹) was much higher than that of panose (0.242 s⁻¹), and hydrolysis of isomaltotriose and nigerose was extremely low. Because Kfla1895 has a strong preference for the α-(1→3)-isomaltosyl moiety and effectively hydrolyzes the α-(1→3)-glucosidic linkage, it should be termed 1,3-α-isomaltosidase. Kfla1896 effectively hydrolyzed isomaltose with liberation of β-glucose, but displayed low or no activity toward CNN and the general GH15 enzyme substrates such as maltose, soluble starch, or dextran. The Kcat/Km for isomaltose (4.81 ± 0.18 s⁻¹ mM⁻¹) was 6.9- and 19-fold higher than those for panose and isomaltotriose, respectively. These results indicate that Kfla1896 is a new GH15 enzyme with high substrate specificity for isomaltose, suggesting the enzyme should be designated an isomaltotetraose glucohydrolase. This is the first report to identify a starch-utilization pathway that proceeds via CNN.

Cyclobis-(1→6)-α-nigerosyl (CNN),3 also known as cyclomaltotetraose or cyclic tetrasaccharide, is a cyclic glucotetraose with alternate α-(1→6)- and α-(1→3)-glucosidic linkages (Fig. 1). This cyclic sugar was first produced from alternan, an alternating linear α-(1→6)-/α-(1→3)-glucan, by treatment with a glycanase termed alternanase, which is produced by Bacillus sp. NRRL B-21195 (1). Nishimoto and co-workers (2–4) subsequently identified three bacterial species (Sporosarcina globispora C11 and N75, formerly known as Bacillus globisporus, and Arthrobacter globiformis A19) that generate CNN from starch. Their ability to produce CNN is derived from two extracellular glycosyltransferases: 6-α-glucosyltransferase (6-GT) and 3-α-isomaltosyltransferase (IMT). 6-GT catalyzes α-(1→6)-glucosyl transfer to the nonreducing terminal glucose unit of α-(1→4)-glucan (GLn) and produces α-glucosyl-(1→6)-GLn (α-isomaltosyl-GLn). IMT utilizes α-isomaltosyl-GLn as both donor and acceptor substrate and catalyzes the α-(1→3) transfer of the α-isomaltosyl moiety of one substrate to the other, resulting in α-isomaltosyl-(1→3)-α-isomaltosyl-GLn formation. Furthermore, IMT catalyzes the intramolecular cyclization of α-isomaltosyl-(1→3)-α-isomaltosyl-GLn, and eventually generates CNN. Currently, it is known that Bacillus sp. NRRL B-21195 produces CNN from maltoligosaccharides using the alternanase and a 6-GT (5, 6). Gene clusters encoding 6-GT and IMT were identified in S. globispora C11 and N75, and A. globiformis A19 (3, 4, 7). Their 6-GT and IMT share high sequence similarity with related enzymes found among the bacterial species. All these enzymes are members of glycoside hydrolase family (GH) 31, which is a sequence-based enzyme classification (8). These studies imply that a common starch metabolic pathway that proceeds via CNN, in which 6-GT and IMT play crucial roles, had been distributed in some bacterial species beyond the genus.

Although CNN-producing enzymes have been identified in some bacteria, whether and how the bacterial species utilize CNN as a carbon source is unclear. Kim et al. (9) reported the purification and characterization of a CNN-degrading enzyme from a lysate of Bacillus sp. NRRL B-21195. This intracellular enzyme hydrolyzed CNN and produced isomaltose via the intermediate α-isomaltosyl-(1→3)-isomaltose. Thus, the authors suggested that CNN is imported into the cells in its cyclic form, as well as cyclomaltodextrins, which are the most widely known cyclic glucans (10). However, the complete CNN degradation pathway is yet unclear because no gene encoding a CNN-degrading enzyme or related proteins, such as a transporter or an isomaltose-degrading enzyme, has been identified.

In the present study, we revealed that the actinobacterium Kribbella flavida NBRC 14399T extracellularly produces CNN from starch, and intracellularly degrades CNN into glucose via isomaltose. K. flavida is a Gram-positive, aerobic, and mesophilic actinomycete isolated from soil or scabby potato, and its genome has been completely sequenced (11). This enabled us to

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2 To whom correspondence may be addressed: Research Faculty of Agriculture, Hokkaido University, kita-9 nishi-9, kita-ku, Sapporo 060-8589, Japan. Tel./Fax: 81-11-388-4603; E-mail: t.tagami@ag.peak.hokudai.ac.jp.

3 The abbreviations used are: CNN, cyclobis-(1→6)-α-nigerosyl; 6-GT, 6-α-glucosyltransferase; ABC, ATP-binding cassette; ESI, electrospray ionization; GH, glycoside hydrolase family; GLn, α-(1→4)-glucan; IMT, 3-α-isomaltosyltransferase; ROK, repressor open reading frame kinase.
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FIGURE 1. Cyclobis-(1→6)-α-nigerosyl.

identify two gene clusters related to CNN production and CNN degradation, respectively, in the genome. CNN degradation is catalyzed by two novel glycoside hydrolases that belong to GH31 and GH15, respectively. This is the first report to demonstrate this unique pathway, which metabolizes starch to glucose via CNN.

Results

Production and Degradation of CNN by K. flavida—K. flavida was cultured using soluble starch as the sole carbon source. The bacterium accumulated some oligosaccharides in the culture supernatant, one of which was a glucoamylase-resistant carbohydrate (Fig. 2A). Its molecular weight was estimated to be 648.2 using electrospray ionization (ESI)-mass spectrometry, and all chemical shifts observed in the 1H and 13C NMR spectra corresponded to those of CNN (1) (data not shown). These results demonstrate that this oligosaccharide was CNN.

The time courses of growth (A600) and CNN concentration in the culture supernatant were monitored (Fig. 2B). The A600 increased during the first 72 h of cultivation and then was decreased upon further cultivation. CNN was not observed in the culture medium until 72 h of cultivation, but further cultivation increased the accumulation of CNN. These observations indicate that K. flavida accumulated CNN in the death phase. The carbohydrate assimilation test showed that K. flavida grew well on CNN at the same level as on glucose and maltose (Fig. 2C), meaning that CNN is a favorable carbon source for this bacterium.

To ascertain whether K. flavida possesses CNN-degrading activity, a cell-free lysate, and a membrane fraction were reacted on CNN (Fig. 2D). Although the membrane fraction did not degrade CNN, the cell-free lysate produced isomaltooligosaccharides, glucose, and an oligosaccharide that may be a linearized CNN. These results indicate that K. flavida intracellularly catabolizes CNN via isomaltooligosaccharides.

Candidate Gene Clusters Related to the Production and Degradation of CNN—A search for genes in the K. flavida genome that encode proteins sharing similarity with IMT of A. globiformis A19 (UniProtKB accession: Q6BD67) was performed using the protein blast. Based upon the search results, we focused on two genetic loci: Kfla_4052 and Kfla_1895.

Kfla_4052 appears to form a gene cluster with Kfla_4051 and Kfla_4053 (Fig. 3A). The 1,108-amino acid GH31 protein encoded by Kfla_4052, designated Kfla4052, shares 79.0% similarity (with 0.4% gaps) calculated using the EMBOSS Water pairwise sequence alignment tool (12) with the IMT of A. globiformis A19. The 948-amino acid protein encoded by Kfla_4051, designated Kfla4051, shares high (78.3%) sequence similarity with the GH31 protein 6-GT of A. globiformis A19. The upstream gene (Kfla_4053) was annotated as encoding a transmembrane regulator: repressor, open reading frame, kinase (ROK) family protein. Both Kfla4051 and Kfla4052 were predicted to possess a signal peptide, indicating that they are extracellular enzymes.

Kfla_1895 appears to form another gene cluster with Kfla_1896 to Kfla_1900 (Fig. 3B). The 723-amino acid GH31 protein encoded by Kfla_1895, designated Kfla1895, shares similarities with the IMT of A. globiformis A19 (46.2% similarity with 17.3% gaps) as well as other IMTs and Kfla4052. These enzymes were predicted to have an extra domain, a family-35 carbohydrate-binding module (CBM35) (8), at the C-terminal side of the catalytic domain, whereas Kfla1895 was predicted to be a single-domain protein. Its upstream gene, Kfla_1896, was annotated as encoding a family-15 glycoside hydrolase. However, this 385-amino acid enzyme, Kfla1896, shared very weak similarity with GH15 enzymes, such as the glucoamylase of Thermoactinomyces vulgaris R-47 (37.1%) and the α,α-trehalase of Mycobacterium smegmatis str. MC2 155 (37.6%) because of high gap scores (25.2 and 15.4%, respectively). Both Kfla1895 and Kfla1896 were predicted to be intracellular pro-
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(A) Kfla_4051 Kfla_4052 Kfla_4053

(B) Kfla_1895 Kfla_1896 Kfla_1897 Kfla_1898 Kfla_1900

(C) Culture (h)

FIGURE 3. Analysis of candidate genes. A and B, two focused gene clusters containing Kfla_4052 and Kfla_1895, respectively. The values below each gene indicate the pairwise alignment score calculated using EMBOSS Water (12). The enzyme abbreviations and their UniProtKB accessions as follows: Ag_6GT, 6-GT of A. globiformis A19 (Q9B3P6); SpN75_6GT, 6-GT of S. globispora N75 (Q84IQ2); Cj_4GT, 4GT of Clostridium jejuni (Q85Z00); SpC11_4GT, 4GT of S. globispora C11 (Q85Z00); SpN75_4GT, 4GT of S. globispora N75 (Q84IQ2); Tv_GA, glucoamylase of T. vulgaris R-47 (O9WKW2); Ms_Tre, α,α-trehalase of M. smegmatis str. MC2 155 (A0R0W9); Sh_GA, glucoamylase of Streptomyces hygroscopicus subsp. limoneus KCCM 11405 (Q15JF7). C, gene expression analysis by semiquantitative RT-PCR. The 16S rRNA encoded by Kfla_R0040 was used as a reference gene.

The expression levels of Kfla_4051, 4052, 1895, and 1896 in K. flavida cells during CNN production were analyzed by semiquantitative RT-PCR (Fig. 3C). All of their transcripts were present at detectable levels in cells cultured for 48 h or more. The expression levels of Kfla_1895 and Kfla_1896 were maintained during 168 h of culture, but those of Kfla_4051 and Kfla_4052 decreased at 168 h compared with their levels from 48 to 96 h.

Enzymatic Properties of Kfla4052, Kfla1895, and Kfla1896—Kfla4051, Kfla4052, Kfla1895, and Kfla1896 were expressed as recombinant proteins in Escherichia coli. All of the recombinant enzymes, except Kfla4051, were produced in a soluble form. Kfla4052, Kfla1895, and Kfla1896 were purified and characterized.

Kfla4052 was reacted on 20 mM panose (α-isomaltosyl-1→4)-glucose and the products were analyzed using TLC (Fig. 4A). The enzyme initially produced glucose and an oligosaccharide with a retention factor similar to that of maltotetraose. Subsequently, the reaction mixture accumulated another oligosaccharide having a retention value similar to that of CNN, and subsequently accumulated isomaltose. The initial rate for hydrolysis of 10 mM CNN, determined by an increase in the concentration of reducing ends, was 11.6 s⁻¹. Kfla1895 activity was the highest at pH 7.9, and the enzyme was stable between pH 6.8 and at least pH 10.7, and at <41 °C. Kfla1895 also hydrolyzed panose into glucose and isomaltose (data not shown); however, the initial rate of panose hydrolysis was 2% of that of CNN, and those of isomaltotriose and nigerose were vanishingly low (Table 1). The s-v plots for the hydrolysis of CNN were fitted to the Michaelis-Menten equation (Fig. 4C) and the kcat and Km values were determined to be 22.3 ± 1.7 s−1 and 7.63 ± 1.38 mM, respectively. The kcat/Km for CNN (2.97 ± 0.34 s−1 mM−1) was 57-fold higher than for panose (Table 2).

The sequence comparison with other GH31 enzymes revealed that two conserved regions, regions A and B, including the catalytic nucleophile and acid/base Asp residues, respectively (13), were also observed in Kfla1895 (Fig. 5A). The site-directed mutations of Asp516 of Kfla1895 to Ala resulted in complete loss of the CNN hydrolyzing activity (Fig. 5B). These results implied that Asp516 of Kfla1895 to Ala resulted in complete loss of the CNN hydrolyzing activity (Fig. 5B). These results implied that Asp516 of Kfla1895 to Ala resulted in complete loss of the CNN hydrolyzing activity (Fig. 5B). These results implied that Asp516 of Kfla1895 to Ala resulted in complete loss of the CNN hydrolyzing activity (Fig. 5B). These results implied that Asp516 of Kfla1895 to Ala resulted in complete loss of the CNN hydrolyzing activity (Fig. 5B). These results implied that Asp516 of Kfla1895 to Ala resulted in complete loss of the CNN hydrolyzing activity (Fig. 5B). These results implied that Asp516 of Kfla1895 to Ala resulted in complete loss of the CNN hydrolyzing activity (Fig. 5B). These results implied that Asp516 of Kfla1895 to Ala resulted in complete loss of the CNN hydrolyzing activity (Fig. 5B). These results implied that Asp516 of Kfla1895 to Ala resulted in complete loss of the CNN hydrolyzing activity (Fig. 5B).

Kfla1896 was reacted on the reaction products from Kfla1895-catalyzed CNN hydrolysis, and the products of this reaction were analyzed using TLC (Fig. 4B, lanes 6 and 7). Kfla1896 completely hydrolyzed isomaltose to glucose and generated another oligosaccharide having a retention value similar to that of panose. Substrate specificity analysis revealed that Kfla1896 displays the highest reaction rate with isomaltose, lower rates with panose and isomaltotriose, but almost no activity with other α-glucobiases (trehalose, kojibiose, nigerose, and maltose), CNN, or polysaccharides (dextran and soluble starch) (Table 1). The isomaltose-hydrolyzing activity was the highest at pH 6.7, and the enzyme was stable between pH 6.5 and at least pH 11.2, and at <35 °C. All s-v plots for the hydrolysis of isomaltose, panose, and isomaltotriose were fitted to the Michaelis-Menten equation (Fig. 4D) and their kinetic parameters were determined (Table 2). Kfla1896 displayed a Kcat/Km value for isomaltose that was similar to those of its other substrates, but a 10-fold higher kcat value for isomaltose than those
for the other substrates. Thus, Kfla1896 exhibited a \( k_{\text{cat}}/K_m \) value for isomaltose that was 6.9- and 19-fold higher than those for panose and isomaltooltriose, respectively. The anomeric configuration analysis of product showed that Kfla1896 produced the /H9252-anomer of glucose (data not shown). Furthermore, Kfla1896 possessed two conserved regions among GH15 enzymes, regions III and V, including the catalytic acid and base glutamic acid residues, respectively (14), and the substitutions of Glu178 and Glu335 of Kfla1896 by Ala markedly decreased or lost the activity (Fig. 5, C and D). These results indicated that the catalytic mechanism of Kfla1896 is identical to those of other GH15 enzymes.

Discussion

Although an enzyme system that produces CNN from starch was found in some bacterial species two decades ago, it had not been clear how these microorganisms use CNN as a carbon source. The present study demonstrated that K. flavida NBRC 14399\(^T\) produces CNN extracellularly from starch and degrades CNN intracellularly to glucose. The proteins involved in the metabolic pathway are encoded by two gene clusters, from Kfla_4051 to Kfla_4053 for production, and from Kfla_1895 to Kfla_1900 for catabolism. Each gene cluster is likely to be regulated by its own ROK family protein, encoded by Kfla_4053 or Kfla_1900, respectively. ROK family proteins are often found as transcriptional repressors for sugar catabolic operons (15). CNN production from starch may be catalyzed by for the other substrates. Thus, Kfla1896 exhibited a \( k_{\text{cat}}/K_m \) value for isomaltose that was 6.9- and 19-fold higher than those for panose and isomaltooltriose, respectively. The anomeric configuration analysis of product showed that Kfla1896 produced the \( \beta \)-anomer of glucose (data not shown). Furthermore, Kfla1896 possessed two conserved regions among GH15 enzymes, regions III and V, including the catalytic acid and base glutamic acid residues, respectively (14), and the substitutions of Glu178 and Glu335 of Kfla1896 by Ala markedly decreased or lost the activity (Fig. 5, C and D). These results indicated that the catalytic mechanism of Kfla1896 is identical to those of other GH15 enzymes.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( \nu ) ( \text{s}^{-1} )</th>
<th>Ratio of ( \nu )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNN</td>
<td>11.6</td>
<td>100</td>
</tr>
<tr>
<td>Panose</td>
<td>2.42 ( \times 10^{-1} )</td>
<td>2.1</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>1.01 ( \times 10^{-2} )</td>
<td>0.0087</td>
</tr>
<tr>
<td>Nigerose</td>
<td>9.90 ( \times 10^{-2} )</td>
<td>0.085</td>
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</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( k_{\text{cat}} ) ( \text{s}^{-1} )</th>
<th>( K_m ) ( \text{mM} )</th>
<th>( k_{\text{cat}}/K_m ) ( \text{s}^{-1} \text{mM}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kfla1895</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNN</td>
<td>22.3 ( \pm 1.7 )</td>
<td>7.63 ( \pm 1.38 )</td>
<td>2.97 ( \pm 0.34 )</td>
</tr>
<tr>
<td>Panose</td>
<td>ND(^a)</td>
<td>ND</td>
<td>0.0524 ( \pm 0.0086 )</td>
</tr>
<tr>
<td>Kfla1896</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isomaltose</td>
<td>11.4 ( \pm 0.3 )</td>
<td>2.38 ( \pm 0.07 )</td>
<td>4.81 ( \pm 0.18 )</td>
</tr>
<tr>
<td>Panose</td>
<td>1.17 ( \pm 0.01 )</td>
<td>1.68 ( \pm 0.06 )</td>
<td>0.699 ( \pm 0.020 )</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>1.07 ( \pm 0.02 )</td>
<td>4.36 ( \pm 0.21 )</td>
<td>0.247 ( \pm 0.008 )</td>
</tr>
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\(^a\) ND, not determined.
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Kfla4052, with possible assistance from Kfla4051. We showed that Kfla4052 produces CNN from panose, demonstrating that this enzyme is a typical IMT (Fig. 4A). We were unable to produce Kfla4051 in our study, but sequence comparison strongly supports that Kfla4051 is a 6-GT. These findings suggest that K. flavida NBRC 14399\textsuperscript{T}, as well as other bacterial species, produce CNN from starch using the well known enzyme system consisting of 6-GT and IMT. The extracellularly produced CNN is thought to be imported into the cells via the sugar ABC transporter encoded by Kfla_1897\textsuperscript{2} and degraded into glucose. The characterizations of Kfla1895 and Kfla1896 reveal that Kfla1895 is a 6-GT, and Kfla1896 is a typical IMT (Fig. 4B). It is speculated that K. flavida produces CNN from starch to secure the carbon source from competitors. Few bacterial species utilizing CNN have been reported so far. On the other hand, gene clusters containing an ROK-family protein, a sugar ABC transporter, a CNN-degrading enzyme that had no sequence similarity to CNN and galactosidase are present in the genomes of Nocardoides sp. JS614 (gene loci: Noca_4630–Noca_4635), Microbispora sp. ATCC PTA-5024 (gene loci: MPTA0524_20930–MPTA0524_20955), Intrasporangium oryzae NRRL B-24470 (gene loci: I42 glucodextranase), and Trueperella pyogenes (gene loci: X956_02355–X956_02380), even though the Kfla1896-like protein is replaced with an oligo-1,6-glucosidase in L oryzae and T. pyogenes, respectively. These bacterial species are expected to catabolize CNN according to the same pathway as K. flavida. Furthermore, Kim et al. previously reported a CNN-degrading enzyme that had no sequence similarity to GH31 enzymes and displayed similar \( k_{cat}/K_m \) values for CNN and panose. This study suggests that another CNN degradation pathway, catalyzed by an enzyme having substrate specificity different from that of Kfla1895, is distributed among the other bacterial species.

The characterizations of Kfla1895 and Kfla1896 reveal that they are novel GH31 and GH15 enzymes, respectively. Kfla1895 effectively hydrolyzes \( \alpha-(1\rightarrow3) \)-glucosidic linkages in both CNN and α-isomaltosyl-(1→3)-α-isomaltose, but the hydrolyzing activities with other substrates having an α-isomaltosyl moiety (isomaltotriose) or an α-(1→3)-glucosidic linkage

![Diagram](http://www.jbc.org/)

**FIGURE 5.** Catalytic residues of Kfla1895 and Kfla1896. A, multiple sequence alignment around conserved regions A and B in GH31. Asp\textsuperscript{451} and Asp\textsuperscript{516} in Kfla1895 were predicted as nucleophile and acid/base residues, respectively. The enzyme abbreviations and their UniProtKB accessions are shown in Fig. 3 and also as follows: Ec_Xyl, E. coli str. K-12 substr. MG1655 α-xylanase (P31434); Ss_AG, Streptomyces simulans α-glucosidase (P0CD66); By_AG, Beta vulgaris α-glucosidase (O04931); Hv_AG, Homo sapiens maltase unit (O43451); Lj_AG, Lactobacillus johnsonii NBRC 13552 α-1,3-glucosidase (C1RT5); Bo_AG, Blautia obeum ATCC 29174 (ASZY13); Sp_AG, Schizosaccharomyces pombe 972h-α-glucosidase (Q9C0Y4); Cj_Xyl, Chaetomium thermophilum var. thermophilum DSM 1495 glucosidase II subunit (O85672); Cj_4GT, C. japonicus Ueda107 α-xylanase (B3PBD9); Cj_4GT, C. japonicus Ueda107 oligosaccharide α-1,4-transglucosylase (Q8RDL1). B, specific activities of Kfla1895 and its mutant enzymes (D451A and D516A), in which Asp\textsuperscript{451} and Asp\textsuperscript{516} were substituted by Ala, respectively. C, multiple sequence alignment around conserved regions III and V in GH15. Glu178 and Glu335 in Kfla1895 were predicted as nucleophile and acid/base residues, respectively. The enzyme abbreviations and their UniProtKB accessions are shown in Fig. 3 and also as follows: Aa_GA, Aspergillus awamori glucoamylase (A2QHE1); Aa_GA, Aspergillus awamori glucoamylase (A2QHE1); Ac_GA, Aspergillus oryzae glucoamylase II (G0SG42); Cj_Xyl, Chaetomium thermophilum var. thermophilum DSM 1495 glucosidase II subunit (O85672); Cj_4GT, C. japonicus Ueda107 α-xylanase (B3PBD9); Cj_4GT, C. japonicus Ueda107 oligosaccharide α-1,4-transglucosylase (Q8RDL1). D, specific activities of Kfla1896 and its mutant enzymes (E178A and E335A), in which Glu\textsuperscript{178} and Glu\textsuperscript{335} were substituted by Ala, respectively. Both multiple sequence alignments were produced using COBALT (33) and rendered using ESPript 3.0 (25).
Two Novel Glycosidases of GH31 and GH15 Degrade CNN

(ngerose) were very low (Table 1). These results indicate that Kfla1895 has a strong preference for the α-(1→3)-isomaltosyl moiety and effectively hydrolyzes the α-(1→3)-glucosidic linkage, and thus should be termed 1,3-moiety and effectively hydrolyzes the region of Kfla1895 and IMTs in the structure-known GH31 enzymes. The characteristic specificity, including α-glucosidase (16), α-xilosidase (17), and α-galactosidase (18), but their reactions share a common feature, liberation of a monosaccharide. Kfla1895 is the only GH31 enzyme to liberate a disaccharide. This difference in product specificities should be attributable to a difference in the structures of their active sites. All GH31 enzymes of known structure have a pocket-shaped active site that is occupied by a monosaccharide moiety at the non-reducing end of the substrate (16). In contrast, it is speculated that Kfla1895 possesses a deep pocket-shaped or groove-shaped active site that accommodates an isomaltosyl moiety. Furthermore, Kfla4052 is expected to have an active-site structure similar to that of Kfla1895 because this enzyme also recognizes an isomaltosyl moiety in its transferring reaction. The sequence comparison suggests that Kfla1895 and all IMTs possess a characteristic region at the loop joining β-strand 2 to α-helix 2 in the catalytic (β/α)$_3$-barrel domain (Fig. 6). Such loop forms part of the rim of the active-site pocket in the structure-known GH31 enzymes. The characteristic region of Kfla1895 and IMTs in β→α loop 2 might contribute to their unique substrate recognition.

The enzymatic properties of Kfla1895 are also novel. Kfla1896 displays strict substrate specificity for isomaltose (Tables 1 and 2). This specificity is similar to that of oligo-1,6-glucosidase (EC 3.2.1.10), which belongs to GH13 (19). However, their reaction mechanisms are different (20). Oligo-1,6-glucosidase follows a double-displacement mechanism, which achieves the hydrolysis of a glucoside with net retention of anomeric configuration. However, GH15 enzymes, including Kfla1896, achieve catalysis using a single-displacement mechanism, which is performed with net inversion. Thus Kfla1896 should be distinguished from oligo-1,6-glucosidase and be termed isomaltose glucohydrolase. The substrate specificity of Kfla1896 is unique among members of GH15. Almost all of the enzymes classified into GH15 are glucoamylases (EC 3.2.1.3), which generally display high substrate specificity for soluble starch. Some bacterial glucoamylases, however, prefer malto-oligosaccharides to soluble starch (14, 21, 22), and only two enzymes, glucodextranase (23) and α,α-trehalase (24), are known to have different substrate specificities. The glucodextranase derived from A. globiformis 142 displays 100- and 19-fold higher activity for dextran and isomaltose, respectively, than for soluble starch (22). Kfla1896 and this glucodextranase have the same regioselectivity for α,1,6-glucosidic linkages but a different preference for the degree of polymerization of their substrates. Mizuno et al. (23) proposed that Gln$^{380}$ and Trp$^{582}$ contributed to the regioselectivity of the glucodextranase. However, their equivalent residues in Kfla1896 were unclear because these enzymes share low sequence similarity. Many GH15 enzymes have some additional domains, such as starch-binding domain of fungal enzymes and a unknown function N-terminal β-sandwich domain of bacterial enzymes, to the catalytic (α/β)$_3$-barrel domain (26), but Kfla1896 is predicted to have only the (α/β)$_3$-barrel domain. It is likely that the strict specificity of Kfla1896 for isomaltose is achieved by the catalytic domain and does not require such additional domains, which generally interact with long-chain substrates at a distance from the active site. Kfla1896 may possess an active-site structure that is different from those of the GH15 enzymes of known structure. Its unique substrate recognition mechanisms can be revealed through other means, like crystal structure analysis.

In conclusion, we elucidated a starch utilization pathway that proceeds via CNN in K. flavida. The intracellular degradation of CNN was catalyzed by two novel glycoside hydrolases, a 1,3-α-isomaltosidase (Kfla1895) of GH31 and an isomaltose glucohydrolase (Kfla1896) of GH15. The identification of the enzymes will expand our understanding of structure-function relationships among glycoside hydrolases.

Experimental Procedures

**CNN Production and Carbohydrate Utilization by K. flavida—K. flavida** NBRC 14399$^T$ was obtained from the Biological Resource Center, National Institute of Technology and Evaluation (Tokyo, Japan). The cells were revived and grown on yeast extract/malt extract (0.4% Bacto yeast extract, 1% Bacto malt extract, and 0.4% glucose) agar according to the protocol supplied with the organism. Liquid yeast extract/malt extract medium was inoculated with a single colony and cultured at 30 °C for 72 h with shaking. The resultant seed culture was diluted 20-fold with CNN-producing medium (0.1% Bacto yeast extract, 0.2% Bacto peptone, 0.1% Bacto beef extract, 0.05% MgSO$_4$.7H$_2$O, and 5% soluble starch) and cultivated at 30 °C for 72 h with shaking. The resultant seed culture was diluted 20-fold with CNN-producing medium (0.1% Bacto yeast extract, 0.2% Bacto peptone, 0.1% Bacto beef extract, 0.05% MgSO$_4$.7H$_2$O, and 5% soluble starch) and cultivated at
30 °C with shaking. Soluble starch was replaced by 5% glucose, maltose, or CNN as a sole carbon source for testing carbohydrate assimilation.

**Purification and Analysis of CNN**—The culture supernatant was obtained by centrifugation (4,500 × g, 10 min, 4 °C). Ethanol was added to the culture supernatant to a final concentration of 80% (v/v), and the resultant precipitate containing soluble starch was removed by centrifugation (12,000 × g, 10 min, 4 °C). The supernatant was dried with a rotary evaporator and the remaining material was redissolved in H₂O. Residual 4 °C). The supernatant was dried with a rotary evaporator and the remaining material was redissolved in H₂O. Residual glutamic acid was obtained by centrifugation (4,500 × g, 10 min, 4 °C), followed by filtration with a Minisart filter (pore size 0.2 μm; Sartorius Stedim Biotech, Gottingen, Germany). The filtrate was desalted using Amberlite MB-4 (Organo Corporation, Tokyo, Japan), concentrated in vacuo, and subjected to preparative HPLC using a Tosoh HPLC system (Tokyo, Japan) equipped with a Cosmosil Sugar-D column (ϕ 10 × 250 mm; Nacalai Tesque, Kyoto, Japan). The sugars were eluted with an isocratic mobile phase of 70% (v/v) acetone at a flow rate of 3 ml/min. The fractions containing only CNN were identified using TLC as described below.

The structure of the purified CNN was confirmed by ESI-MS analysis using an Exactive mass spectrometer (Thermo Fisher Scientific; Waltham, MA) and NMR analysis using a Bruker AMX500 (1H, 500 MHz; 13C, 126 MHz) spectrometer (Bruker, Billerica, MA). The concentration of the purified CNN was determined using the phenol-sulfuric acid method (27) with glucose as a standard. The concentration of CNN in the culture supernatant was determined using a high-performance anion exchange chromatography system equipped with a pulsed amperometric detector and a CarboPac PA1 column (ϕ 250 mm; Dionex, Sunnyvale, CA). The solution obtained prior to the elimination of maltotriosaccharides was subjected to the analysis. The sugars were eluted with an isocratic mobile phase of 0.8 M NaOH at a flow rate of 0.8 ml/min. Sorbitol (100 μM) was used as an internal standard. TLC was performed using a Silica Gel 60 aluminum plate (Merck Millipore, Darmstadt, Germany). Carbohydrates were developed twice using a solvent system of acetonitrile/H₂O (75:25; v/v), and visualized by heating with a reagent containing 0.3% 1-naphtol and 5% sulfuric acid in methanol.

**CNN-degrading Activity of K. flavida—**K. flavida cells (A₅₅₀ = 30) cultured using CNN-producing medium were disrupted with a mortar and pestle in liquid nitrogen and suspended in 1 ml of 20 mM HEPES-NaOH (pH 7.5). A portion of the suspension (500 μl) was vortexed well and centrifuged (13,700 × g, 10 min, 4 °C). The resultant supernatant was isolated as a cell-free lysate, and the precipitate was re-suspended in 500 μl of 20 mM HEPES-NaOH (pH 7.5) as a membrane fraction. The cell-free lysate or the membrane fraction (190 μl) was mixed with 100 mM CNN (10 μl) and incubated at 30 °C for 24 h. These reaction mixtures were analyzed using TLC.

**Semiquantitative RT-PCR—**Total RNA from K. flavida cells cultured in CNN-producing medium was isolated using the NucleoSpin RNA kit (Takara Bio, Kusatsu, Japan). Each total RNA sample (200 ng) was subjected to reverse transcription using PrimeScript reverse transcriptase with random 6-mer oligonucleotide primers (PrimeScript RT-PCR Kit; Takara Bio). The resultant reaction mixtures (1 μl) were used as templates for subsequent PCR. TaKaRa Ex TaqHS DNA polymerase (Takara Bio) was used in assays to detect mRNA encoding Kfla_4051, Kfla_4052, Kfla_1895, Kfla_R0040, and TaKaRa Ex TaqDNA polymerase (Takara Bio) was used in assays to detect mRNA encoding Kfla_1896. The oligonucleotide primers used for the PCR were as follows: 5′-CCTTACCTGACGCTGAGGAG-3′ and 5′-CCGGTACCGTCACTTGTGA-3′ for Kfla_4051 (775 bp), 5′-GGCGAGTACAAAGGCAAC-3′ and 5′-GTAATCCGCGTGGACCTGTG-3′ for Kfla_4052 (783 bp), 5′-CGAGATGCTGACGACGGTGA-3′ and 5′-TACCCCA-CGACTGAACAGC-3′ for Kfla_1895 (776 bp), and 5′-TACCGGACATGTCGGTGTGTTGTC-3′ and 5′-GTCGGCGAGGATCTAGGA-3′ for Kfla_1896 (723 bp). The numbers in parentheses indicate the sizes of their amplified DNA fragments. The PCR mixtures supplemented with 1 × Loading Buffer (Takara Bio) (7 μl total volume) were subjected to agarose gel electrophoresis, and the amplified DNA fragments were detected with ethidium bromide under a UV lamp.

**Cloning and Construction of Expression Plasmid—**The genome DNA of K. flavida was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Open reading frames were amplified by PCR from the genomic DNA using the following primers: 5′-GTAACCGCTTGCAGAGGTCAC-3′ and 5′-ATCTTTGGGACAGCCGTCGCCCA-3′ for Kfla_4051, 5′-GCAGACAGAAGGTCGATG-3′ and 5′-GATCGACGACGCGAAGGGC-3′ for Kfla_4052, 5′-ACCGCATGTACCTGTACCTC-3′ and 5′-ACAATAGGCGGCTGACC-3′ for Kfla_1895, and 5′-AGCAACAGTGCACGCGTAC-3′ and 5′-CGTACGCCGTGGTCGTGATA-3′ for Kfla_1896. PCR was performed by using KOD-Plus-DNA polymerase or KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan). Each PCR product was ligated into the EcoRV site of the plasmid pBluescript II SK(+) (Agilent Technologies, La Jolla, CA) using Ligation Kit version 2 (Takara Bio), and the resulting plasmids were propagated in E. coli DH5α. The sequences were analyzed by FASMCo, Ltd. (Atsugi, Japan).

The proteins encoded by the genes designated Kfla4051, Kfla4052, Kfla1895, and Kfla1896 were heterologously expressed in E. coli as N-terminal His-tag fusion proteins. DNAAs encoding the putative mature regions of Kfla4051 (Val²⁵-Phe⁸⁴⁹), Kfla4052 (Gly¹³⁵-Pro¹₁⁶⁸), Kfla1895 (Met¹-Ser²⁷³), or Kfla1896 (Met¹-Ala³⁴⁸), which were predicted using the SignalP 4.1 server (28), were amplified by PCR using PrimeSTAR HS (Takara Bio) or KOD-plus-Neo (Toyobo) DNA polymerase and the pairs of primers containing NdeI or HindIII sites (underlined) as follows: 5′-AAAACATATGGTGTTGCGAGGTTCGGATCTTTGTTGCAGACGGCAG-3′ and 5′-TTTAAGCTTTGCTAAGACCCGGCGTCTGCC-3′ for Kfla_4051, 5′-AAACATATGCGGCGGCTGCTT-3′ and 5′-TTTGGGACACCCGGTGGTGTCG-3′ for Kfla_4052, 5′-AAACATATGCGGCGGCTGCTT-3′ and 5′-TTTGGGACACCCGGTGGTGTCG-3′ for Kfla_1895, and 5′-AAACATATGCGGCGGCTGCTT-3′ and 5′-TTTGGGACACCCGGTGGTGTCG-3′ for Kfla_1896.
CATATGACCCACCTCCGCGGGGACA-3′ and 5′-TTTAA-GGTTTCCAGGGCTCTTGGCCGC-3′ for Kfla 1896. Each PCR product was digested with Ndel and HindIII and inserted between the Ndel and HindIII sites of plasmid vector pET-28a (Merck Millipore).

The site-directed mutations were performed with PCR using PrimeSTAR mutagenesis basal kit (TaKaRa Bio). The primers, of which underlined nucleotides indicates the mutated codons, are as follows: 5′-AAAGCCCGGGCGCGACGCAGCC-TGG-3′ and 5′-GCGGCCCACCGGTCTTGATTCGTC-GAC-3′ for Kfla1895 Asp516 → Ala mutation, 5′-GCGGCCCACCGGTCTTGATTCGTC-GAC-3′ and 5′-GTGCTCCGCCACCTGCTGAGCATCGG-3′ and 5′-GACCTGCGCCGGCAGGTCCGAGGGATGCC-3′ for Kfla1896 Glu178 → Ala mutation, and 5′-CTGCCGCGCCGGCAGGTCCGAGGGATGCC-3′ and 5′-GACCTGCGCCGGCAGGTCCGAGGGATGCC-3′ for Kfla1896 Glu355 → Ala mutation.

Production, Purification, and Characterization of Kfla4052—E. coli Rosetta (DE3) cells (Merck Millipore) were transformed with the pET-28a plasmid harboring the Kfla4052 gene. A transformant was cultured overnight at 30 °C in LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, and 0.5% NaCl) containing 50 mg/liter of kanamycin and 30 mg/liter of chloramphenicol. The resultant seed culture was diluted 20-fold with TB medium (1.2% Bacto tryptone, 2.4% Bacto yeast extract, 0.5% NaCl) and incubated at 37 °C for 5 h. The cell-free lysate obtained by centrifugation (12,000 × g, 10 min, 4 °C), suspended in Buffer-A (20 mM sodium phosphate buffer (pH 7.5) and 0.3 M NaCl) supplemented with 0.1% Triton X-100, was loaded onto a Resource S column (20 ml; GE Healthcare) and then loaded onto a Ni-chelating Sepharose Fast Flow column (GE Healthcare, Buckinghamshire, England). After the column was washed with 20 mM imidazole in Buffer A, the adsorbed proteins were eluted with 500 mM imidazole in Buffer A. The active fractions were dialyzed against Buffer B (20 mM sodium phosphate buffer (pH 5.5)) and then loaded onto a Resource S column (6 ml; GE Healthcare). After the column was washed with 0.3 M NaCl in Buffer B, the adsorbed proteins were eluted with a linear gradient of 0.3 to 1 M NaCl in Buffer B. Electrophoretically homogeneous Kfla4052 was dialyzed against Buffer B and concentrated using a Vivaspin 20−50K (GE Healthcare). The concentration of the purified Kfla4052 was determined using its absorbance at 280 nm and the theoretical extinction coefficient (216,010 M⁻¹ cm⁻¹) calculated using ProtParam server (29).

Kfla4052 activity was measured at 37 °C in a standard reaction mixture containing 40 mM MES-NaOH (pH 6.5), 1 mM CaCl₂, 10 mM panose, and Kfla4052, which was diluted to an appropriate concentration using 20 mM MES-NaOH (pH 6.5) containing 0.3% Triton X-100. The reaction mixture (50 µl) was collected at 10 min and the reaction was stopped by treatment at 100 °C for 1 min. The solution was diluted 3-fold with 2 mM Tris-HCl (pH 7.0), and the concentration of glucose was measured using a mutarotase-glucose oxidase method (30). One unit of Kfla4052 was defined as the amount of enzyme that liberates 1 µmol of glucose/min from panose under the standard reaction conditions.

The effect of pH on the activity was investigated under the standard assay conditions but using 40 mM sodium acetate buffer (pH 3.5, 4.5, and 5.4), 40 mM MES-NaOH (pH 6.0 and 6.5), 20 mM MOPS-NaOH (pH 7.0 and 7.7), 20 mM HEPES-NaOH (pH 8.2 and 8.5), or 40 mM glycine-NaOH (pH 9.0 and 10.0). For measurements of pH stability, Kfla4052 was incubated in 0.3% Triton X-100 and 20 mM glycine-HCl (pH 2.4 and 3.2), sodium acetate buffer (pH 3.9, 4.5, and 5.3), MES-NaOH (pH 6.0), MOPS-NaOH (pH 7.1), HEPES-NaOH (pH 7.9), glycine-NaOH (pH 9.1 and 10.1), or CAPS-NaOH (pH 11.1, 11.4, and 11.8) buffer at 4 °C for 24 h. Residual activity was measured under standard assay conditions. For thermostability measurements, Kfla4052 was kept at 30−70 °C for 15 min, and then its residual activity was measured under standard assay conditions. The stable region was defined as the pH or temperature range exhibiting residual activity of more than 90%.

Production and Purification of Kfla1895, Kfla1896, and Their Mutant Enzymes—E. coli Rosetta (DE3) cells were transformed with pET-28a plasmids harboring the Kfla1895 gene, the Kfla1896 gene, or their mutant gene. The production procedures were the same as those used to produce Kfla4052, except that their inductions were performed by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside in LB medium at 20 °C for 6−16 h. The enzymes were purified with a nickel chelating Sepharose column using Buffer C (20 mM sodium phosphate buffer (pH 7.0) and 0.3 M NaCl) as a basal buffer. Electrophoretically homogeneous Kfla1895, Kfla1896, or their mutant was dialyzed against 20 mM sodium phosphate buffer (pH 7.0 or 8.0, respectively) and concentrated using Vivaspin 20−50K or −30K. The concentrations of the purified enzymes were determined using their absorbances at 280 nm and theoretical extinction coefficients, calculated using ProtParam server, of 192,280 and 112,410 M⁻¹ cm⁻¹, respectively.

Characterization of Kfla1895, Kfla1896, and Their Mutants—Kfla1895 activity was measured at 35 °C in a standard reaction mixture containing 80 mM sodium phosphate buffer (pH 8.0), 2 mM CNN, and Kfla1895 and its derivatives, which were diluted to an appropriate concentration using 20 mM sodium phosphate buffer (pH 7.0) containing 0.1% Triton X-100. Reaction mixtures (50 µl) were collected at 3 and 10 min, and the reactions were stopped by treatment at 80 °C for 2 min. The solution was diluted 10-fold with H₂O, and the concentration of reducing sugar was measured using the 2,2'-bicinchoninate method (31) with glucose as the standard. One unit of Kfla1895 was defined as the amount of enzyme that liberates 1 µmol of reducing sugar/min from CNN under the standard reaction conditions.

Kfla1896 activity was measured at 35 °C in a standard reaction mixture containing 40 mM sodium acetate buffer (pH 6.0), 2 mM isomaltose, and Kfla1896 and its derivatives, which were diluted to an appropriate concentration using 20 mM sodium phosphate buffer (pH 8.0) containing 0.1% Triton X-100. The
reaction was stopped at 10 min by 3-fold dilution with 2 M Tris-HCl (pH 7.0), and the concentration of glucose was measured using the mutarotase-glucose oxidase method. One unit of Kfla1896 was defined as the amount of enzyme that hydrolyzed 1 μmol of isomaltose per min under standard reaction conditions. The effect of pH (from pH 2.6 to 10.3) on enzymatic activity was investigated under standard assay conditions, except using Britton-Robinson buffer (a mixture of 40 mM acetic acid, 40 mM phosphoric acid, and 40 mM boric acid adjusted to the appropriate pH with 0.2 N NaOH) as the reaction buffer. To measure pH stability, Kfla1895 or Kfla1896 was incubated in 10-fold-diluted Britton-Robinson buffer (pH 3.1 to 11.2) at 4 °C for 24 h, and then its residual activity was measured under standard assay conditions. To measure thermostability, Kfla1895 or Kfla1896 were kept at 25 °C–55 °C for 15 min, and then its residual activity was measured under standard dilution conditions. The stable region was defined as the pH or temperature range exhibiting residual activity of more than 90%.

Substrate specificity was analyzed by measuring the initial rates of hydrolysis of various substrates under standard reaction conditions. Potential Kfla1895 substrates, tested at 10 mM concentration, included CNN, isomaltotriose, panose, and nigerose. Potential Kfla1896 substrates included isomaltose, isomaltotriose, panose, maltose, kojibiose, nigerose, trehalose, and CNN at 10 mM concentrations, as well as 2 mg/mL of dextran 10 and soluble starch. For both enzymes, initial reaction rates with CNN and the others were determined using the rate of the increase in reducing sugar content and glucose, respectively.

The kinetic parameters, $k_{cat}$ and $K_m$, for substrate hydrolysis were calculated from $v$-$s$ plots by fitting to the Michaelis-Menten equation using KaleidaGraph 3.6 software (Synergy Software, Reading, PA). The substrate concentrations were 2–40 mM CNN for Kfla1895 and 1–20 mM isomaltose, isomaltotriose, and panose for Kfla 1896. Because the $K_m$ value of Kfla1895 for panose was so large, only the $k_{cat}/K_m$ value was determined from the Lineweaver-Burk plots at concentrations from 1.7 to 10 mM. Each measurement was made in triplicate.

The anemic form of the product resulting from Kfla1896-catalyzed hydrolysis was determined by using 4-nitropheny 1-glucopyranoside as a substrate. The enzyme (1.8 units/mL) was incubated with 7.5 mM 4-nitropheny 1-glucopyranoside in 5 mM sodium phosphate buffer (pH 8.0) at 30 °C for 20 min. The anemic form of glucose produced by the reaction was analyzed by HPLC using a previous report procedure (32).

Author Contributions—T. T. and M. O. designed the research and generated the manuscript. E. M. characterized Kfla4052 and J. S. performed the experiments shown in Fig. 2. T. T. performed all other experiments. T. I. and A. K. provided technical assistance and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

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References


Two Novel Glycoside Hydrolases Responsible for the Catabolism of Cyclobis-(1→6)-α-nigerosyl
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