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Cloning, Sequencing and Expression of the Genes Encoding Cyclic α-Maltosyl-(1→6)-maltose Hydrolase and α-Glucosidase from an Arthrobacter globiformis Strain

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Abstract: The gene encoding for a novel hydrolase, cyclic α-maltosyl-(1→6)-maltose [CMM, cyclo-{(1→6)-α-D-Glc}p-(1→4)-α-D-Glc-(1→3)-α-D-Glc-(1→6)-maltose (CMMase), was cloned from the genomic library of Arthrobacter globiformis M6 and designated cmmF. The gene consisted of 1,353 bp encoding a protein of 450 amino-acids with a calculated molecular mass of 49,344 Da. The deduced amino-acid sequence showed similarities to cyclodextrinase, maltogenic amylase and neopullulanase. On the other hand, the complete sequence of the α-glucosidase gene (cmmB), which encodes an enzyme involved in the degradation of CMM, revealed that the gene consisted of 1,704 bp encoding a protein of 567 amino-acids with a calculated molecular mass of 63,014 Da. The four conserved regions common in the α-amylase family enzymes were also found in CMMase and α-glucosidase, indicating that these enzymes should be assigned to this family. The DNA sequence of 5,675 bp analyzed in this study contained another four open reading frames (ORFs), designated cmmC, cmmD, cmmE and cmmG, downstream of cmmB. CmmC, cmmD and cmmE were expected to encode proteins concerned with incorporation of CMM via cell membrane. CmmG was expected to encode a transcriptional regulator protein. CMMase gene, α-glucosidase gene and another four ORFs formed a gene cluster together with 6-α-maltosyltrasferase gene (cmmA) which encodes a CMM-forming enzyme, namely cmmABCDEFG. The results of gene analysis suggested that A. globiformis M6 has a unique starch utilization pathway via CMM.

Key words: cyclic α-maltosyl-(1→6)-maltose, α-1,6 linkage, Arthrobacter globiformis, CMM hydrolase, α-glucosidase

It is known that bacterial enzymes produce cyclic glucans. Cyclodextrin (CD, cyclic α-1,4-glucan), consisting of 6 glucose units or more, is produced from starch by cyclomaltdextrin glucanotransferase (CGTase, EC 2.4.1.19).1,2 Cyclodextran (cyclic α-1,6-glucan, 7–9 glucose units) is produced from dextran by an extracellular enzyme, called cyclomaltdextrin oligosaccharide glucanotransferase [EC 2.4.1.248], from Bacillus circulans T-3040.2,3 All of these cyclic glucans have homogeneous linkages in their structure. Côté and co-workers first reported that a cyclic tetrasaccharide consisting of α-D-glucose, cyclo-{(1→6)-α-D-Glc}p-(1→4)-α-D-Glc-(1→3)-α-D-Glc-(1→6)-α-D-Glc-(1→3)-α-D-Glc-(1→1), was produced from a dextran-like polysaccharide, alternan, by its degrading enzyme.3,4 This cyclic oligosaccharide was designated cycloalternan (CA). Recently, we discovered two novel enzymes, 6-α-glucosyltrasferase and 3-α-isomaltosyltrasferase, in Bacillus globisporus, and succeeded in the mass production of this saccharide from starch by joint reaction of the two enzymes.5–7)

During the course of our screening for microorganisms that produce nonreducing oligosaccharides from starch, we obtained bacterial strain Arthrobacter globiformis M6 from soil.8) This strain produces nonreducing oligosaccharides from starch. Structural analysis showed that the nonreducing oligosaccharide produced by this strain is a novel cyclic tetrasaccharide that differs from CA in terms of glycoside linkages. This cyclic tetrasaccharide, called cyclic α-maltosyl-(1→6)-maltose [CMM, cyclo-{(1→6)-α-D-Glc}p-(1→4)-α-D-Glc-(1→6)-α-D-Glc-(1→4)-α-D-Glc-(1→1)], has a unique structure. Its four glucose residues are joined by alternate α-1,4 and α-1,6 linkages. We previously purified the CMM-forming enzyme, 6-α-maltosyltrasferase (6 MT), from a culture supernatant of A. globiformis M6.9) It was found that 6MT catalyzes both intermolecular and intramolecular α-1,6-maltosyl transfer reactions to synthesize CMM from starch. Furthermore, we previously reported that the two enzymes, CMM hydrolase (CMMase) and α-glucosidase, were applied to these B. globisporus enzymes.10,11) Here we report the cloning, sequencing and expression of the genes encoding cyclic α-maltosyl-(1→6)-maltose hydrolase (CMMase) and α-glucosidase (α-glucosidase) from A. globiformis M6.
Maltoligosaccharides were prepared in our laboratory. Mase hydrolyzed CMM to zymes involved in the degradation of CMM (Fig. 1). CM-

Saccharides produced from these genes. Frames (ORFs). We also report the amino-acid sequences deduced from these genes.

Methylated CMM to glucose. On the other hand, α-glucosidase degraded α-maltosyl-(1→6)-maltose and maltose to glucose; however, it did not degrade CMM. When CMMase and α-glucosidase existed simultaneously in the reaction mixture containing CMM, glucose was detected as the final product. It was found that CMM was degraded to glucose by synergistic action of CMMase and α-glucosidase.

Although we purified and characterized CMMase and α-glucosidase, nucleotide sequences and deduced amino-acid sequences of these enzymes were not defined. Therefore, we tried to clone CMMase and α-glucosidase genes. In our previous experiment, we cloned a 6MT gene designated cmmA (AB190187) and an incomplete gene designated cmmB (AB190187) which was located just downstream of cmmA. The four conserved regions that are common in glycoside hydrolase family 13 (GH 13) enzymes1,12 were found in cmmB; hence we considered the possibility that cmmB might be a partial nucleotide for α-glucosidase which we purified from cell-free extract of A. globiformis M6. In the case of cyclodextrins (CDs), the gene for CD-degradation enzyme (CDase) is located downstream of the gene for the extracellular CD-synthesis enzyme (CGTase).13 Hence we hypothesized that DNA fragments which contain CMMase or an unknown part of cmmB were isolated by this reverse-phase high-performance liquid chromatography (HPLC).

The purified CMMase from A. globiformis M6 strain was digested with 2 µg/mL of modified trypsin (Promega Corporation, Madison, USA) for 14 h at 37°C. The reaction mixture was loaded on a µRPC C2/C18 SC2.1/10 column (0.21 × 10 cm, GE Healthcare UK Ltd., Amersham place, UK) equilibrated with 0.065% trifluoroacetic acid (solution A) and the adsorbed peptides were eluted with a linear gradient of 100% solution A to 100% solution B (0.055% trifluoroacetic acid, 80% acetonitrile) at a flow rate of 0.1 mL/min. Fourteen kinds of peptide fragments were isolated by this reverse-phase high-performance liquid chromatography (HPLC).

**DNA preparation.** Chromosomal DNA was isolated by the method of Saito and Miura.14 Purification of the plasmid DNA was carried out by the method of Birnboim and Doly.15 Oligonucleotides for the PCR primer and sequencing were purchased from Sigma-Aldrich Japan Co. (Ishikari, Japan). Unless otherwise specified, all DNA manipulations were performed by standard methods, as described by Sambrook et al.16

**Nucleotide sequencing.** The nucleotide sequences were analyzed using a DNA sequencer (CEQ8800XL; Beckman Coulter, Inc., Fullerton, USA) with a dye terminator cycle sequencing kit (Beckman Coulter, Inc.). Both strands of the DNA were sequenced, and the resulting data were analyzed using the GENETYX-WIN program (GENETYX CORPORATION, Tokyo, Japan).

**Cloning of a CMMase DNA fragment.** Polymerase chain reaction (PCR) was carried out to obtain a partial fragment of the CMMase gene. A. globiformis M6 chromosomal DNA was used as the template for the PCR. The sequence of the sense primer for the PCR was 5'-GAYCARGONATCG AYGGNTTG-3' (R: A or G; Y: C or T; N: A, C, G, or T) corresponding to the amino-acid sequence of a peptide fragment, DGIDGW (CM-10 in Fig. 2). The sequence of the antisense mix primer for the first PCR was 5'-GTRRTCCAN GAYTCYCTRTTCAA-3' corresponding to the amino-acid sequence of a peptide fragment, WNESWNT (CM-8 in Fig. 2). The temperature program for each cycle was 98°C for 20 s, 55°C for 30 s and 72°C for 2.0 min. After a 1.0 min heat treatment at 98°C for DNA denaturing, 30 cycles were run. The amplified DNA fragment, of about 0.5 kb, was purified by gel electrophoresis and then cloned into a pCR-Script SK + vector. DNA sequencing showed that the 495 bp fragment (PCR-1 in Fig. 3) was a portion of the CMMase gene (nucleotide positions 4,297 to 4,792 in Fig. 2).

**Materials and Methods**

**Saccharides.** CMM, α-maltosyl-(1→6)-maltose and maltotriosaccharides were prepared in our laboratory.

**Microorganisms and cultivation.** A. globiformis M6 was the DNA donor for gene cloning. This strain was cultivated as previously described.10 Escherichia coli XL10-Gold (Stratagene Cloning Systems, Inc., La Jolla, USA) and JM109 (Takara Bio Inc., Ohtsu, Japan) were used as the host for the gene cloning. E. coli BL21 (DE3) (Novagen Inc., Darmstadt, Germany) was used for gene expression. The E. coli cells were grown in Luria broth containing 1.0% tryptone (BD Diagnostic Systems, Sparks, USA), 1.0% yeast extract (BD Diagnostic Systems) and 0.5% NaCl at 37°C (cloning) or 27°C (expression). When necessary, ampicillin (100 µg/mL) or kanamycin (20 µg/mL) was added to the medium.

**Plasmids.** The plasmids pCR-Script SK + and pBluescript II SK+ (Stratagene Cloning Systems, Inc.) were used as a vector for gene cloning. The plasmid pRSETA (Invitrogen Corporation, Carlsbad, USA) was used as an expression vector.
Gene cloning. The genomic DNA from *A. globiformis* M6 was digested with *ApaI* (Takara Bio Inc.). Fragments with a molecular size of about 5.5 kbp were separated and purified from the agarose gel using a QIAEX II Gel Extraction kit (Qiagen GmbH, Hilden, Germany). The fragments were ligated into the *ApaI* site of pBluescript II SK+*. The constructed genomic DNA library was screened by colony hybridization (Roche Diagnostics, Basel, Switzerland). Colony hybridization was performed according to the manufacturer’s protocol. One positive clone containing a DNA fragment, T-1 (nucleotide positions 1 to 5,675 in Fig. 2), was obtained. The plasmid which contains the T-1 fragment was designated pBlue-T1.

Expression of CMMase gene (cmmF) in *E. coli*. Plasmid pBlue-T1 was used as the template. PCR amplification was performed using the two synthetic primers, 5′-GGCATATGGCCGTCGACGGATCC-3′ (the underlined sequence is the position of an *NdeI* site, nucleotide positions 3,724 to 3,741. In Fig, 2, the *GTCGAC* initiation codon was altered to the *ATG* initiation codon.) and 5′-CCGAATTTCTCACGCGAGCTCCC-3′ (the underlined sequence is the position of an internal *EcoRI* site, nucleotide positions 5,062 to 5,076). The PCR product was digested with *NdeI* and *EcoRI*, and then ligated into pRSETA cleaved with the same restriction enzymes. *E. coli* BL21 (DE3) was then transformed using the ligated plasmid (called pRSET-CMMase). Several ampicillin-resistant clones were selected, and the nucleotide sequence of the plasmid was determined. The resulting positive transformant was cultured in Luria broth (150 mL) supplemented with ampicillin (100 μg/mL) and underwent vigorous shaking at 27°C. At 0.6 *Aoo*, isopropyl-thio-β-D-galactopyranoside was added to the culture (0.4 mM) and then incubation was continued for another 3 h. Cells were harvested by centrifugation (10,000 × G for 10 min) and washed with 50 mM sodium acetate buffer, pH 6.0. The washed cells were resuspended in lysozyme solution (100 μg/mL, in 50 mM sodium acetate buffer, pH 6.0) and incubated at 37°C for 1 h. The lysozyme-treated cell suspension was sonicated for 2 min (Ultrasonic Homogenizer UH-600, SMT Co., Ltd., Tokyo, Japan). After the sonication, the cell suspension was centrifuged for 10 min. The supernatant was used as enzyme solution.

Expression of α-glucosidase gene (cmmB) in *E. coli*. The genomic DNA from *A. globiformis* M6 was used as the template. PCR amplification was performed using the two synthetic primers, 5′-GGCATATGGCCGTCGACGGATCC-3′ (the underlined sequence is the position of an *NdeI* site, nucleotide positions 1 to 607 in Fig. 2). In Fig. 2, the *GTCGAC* initiation codon was altered to the *ATG* initiation codon.) and 5′-CCQAATTTCTCACGCGAGCTCCC-3′ (the underlined sequence is the position of an internal *EcoRI* site, nucleotide positions 1,689 to 1,704). The subsequent experiment was performed in a manner identical with that of the CMMase gene described above.

Purification of recombinant CmmB. Purification of CmmB expressed in *E. coli* was carried out according to the method previously described. Unless otherwise stated, all the purification procedures were done at 4°C. The molecular mass of the purified protein was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on a 5 to 20% gradient gel according to the method of Laemmli. Enzyme assay. The enzyme activity of CMMase and α-glucosidase was examined as described in a previous paper.

HPLC analysis. HPLC analysis was performed using an LC-10AD pump, an RID-10A refractive index monitor, and a C-R7A data processor (Shimadzu Corporation, Kyoto, Japan) equipped with an ODS AQ-303 column (0.46 × 25 cm; YMC Co., Ltd., Kyoto, Japan) and eluted with water at a flow rate of 0.5 mL/min at 40°C.

RESULTS AND DISCUSSION

Gene cloning. The amino-acid sequence analysis found fourteen internal sequences, CMM-1 to CMM-14, for CMMase (Fig. 2). During several trials of PCR using primers designed on the basis of the internal amino-acid sequences, we succeeded in amplifying a 495 bp fragment of the CMMase gene from the genomic DNA of *A. globiformis* M6. The amino-acid sequence encoded by this DNA fragment (nucleotide positions 4,297 to 4,792 in Fig 2) contained five internal sequences, CMM7, CMM9, CMM11, CMM12 and CMM14, of CMMase. Hence we concluded that the DNA fragment was part of the CMMase gene. To obtain the full-length CMMase gene, we screened a genomic DNA library of *A. globiformis* M6 by colony hybridization using a 495 bp fragment (PCR-1 in Fig. 3) of the CMMase gene as a probe. As a result, one positive clone carrying a 5,675 bp fragment was obtained. Six open reading frames (ORFs) were found in the fragment. The nucleotides from 1 to 547 of the ORF-1 located at 5′-region of the fragment completely corresponded with the 3′-region of *cmmB* which we previously cloned as the gene located downstream of the 6MT gene designated *cmmA*. Hence, we named the six ORFs, from 5′-region to 3′-region, *cmmB*, *cmmC*, *cmmD*, *cmmE*, *cmmF* and *cmmG*. These nucleotide sequence data have been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB478693 (*cmmB*), AB478694 (*cmmC*), AB478695 (*cmmD*), AB478696 (*cmmE*) and AB478697 (*cmmF*) and AB478698 (*cmmG*).

The α-glucosidase gene. Nucleotide positions 1 to 547 in Fig. 2 completely matched the 3′-region of *cmmB*. Hence we concluded that nucleotide positions 1 to 607 were the 3′-region of *cmmB*. This gene was expected to encode a protein with 567 amino acid residues (calculated molecular mass, 63,014 Da). The deduced amino-acid sequence contained all of the N-terminal and internal sequences of α-glucosidase which we previously purified from cell-free extract of *A. globiformis* M6.

The *cmmB* gene was expressed in *E. coli* BL21 (DE3) and the product was purified by hydrophobic column chromatography and anion exchange column chromatography. CmmB was finally purified to yield a 63 kDa protein on SDS-PAGE as a single band with specific activity of 8.3 U/mg. The N-terminal sequence of the purified protein was SHTIER, identical with the amino-acid sequence from the 2nd to the 7th of α-glucosidase which we had purified.
Fig. 2. Continued on next page.
Fig. 2. Nucleotide sequences of cmmB, cmmC, cmmD, cmmE, cmmF and cmmG and the deduced amino-acid sequences.

Numbers on the right side of the sequences denote nucleotide (upper) and amino-acid (lower) positions. The potential SDs are lined up above the nucleotide sequences. The restriction enzyme (ApaI) positions are boxed. The N-terminal and internal amino-acid sequences of β-glucosidase and CMMase from A. globiformis M6 are underlined.
from the cell-free extract of *A. globiformis* M6 previously. We purified recombinant CmmB from *E. coli* BL21 (DE3) harboring *cmmB*, because it was unable to distinguish between the activity of CmmB and that of intrinsic α-glucosidase. The purified recombinant CmmB showed the same substrate specificity as the α-glucosidase from *A. globiformis* M6. In addition, it showed the same properties, namely, optimum pH and optimum temperature, as the α-glucosidase from *A. globiformis* M6 (data not shown). Considering these results, we concluded that *cmmB* is the gene encoding for the α-glucosidase which we previously purified from the cell-free extract of *A. globiformis* M6.

Homology searches performed with the BLASTP program using a non-redundant protein sequence database showed that the deduced amino-acid sequences showed 51% identities to the α-glucosidase from *Thermomonospora curvata*. The four conserved regions that are common in GH 13 family enzymes were also found in the α-glucosidase from *A. globiformis* M6, indicating that this enzyme should be assigned to this family.

**The CMMase gene.**

As shown in Fig. 2, the CMMase gene (*cmmF*) extended from the GTG initiation codon at position 3,604 to the TGA stop codon at position 5,076, with the potential Shine-Dalgarno sequence (SD), GAAGGA, from position 3,708 to position 3,713. CMMase encoded a protein with 450 amino acid residues. The molecular mass of the gene product was calculated to be 49,344 Da, in agreement with the 48.6 kDa of the purified enzyme (SDS-PAGE). The deduced amino-acid sequence contained all of the N-terminal and internal sequences of CMMase.

The CMMase gene was expressed in *E. coli* BL21 (DE3) under the control of the T7lac promoter of the vector, pRSETA. Enzyme solution prepared as cell-free extract was incubated with CMM and then analyzed by thin-layer chromatography (TLC) (Fig. 4). The reaction mixture showed production of oligosaccharide (saccharide 1 on lane 5) from CMM, which indicates the same *Rf* value as that of α-maltosyl-(1→6)-maltose (Fig. 4). From the comparative analysis with authentic sugars by reverse-phase HPLC, we concluded that saccharide 1 was α-maltosyl-(1→6)-maltose.

The cell-free extract (250 μL) was added to the substrate solution (250 μL) containing 2.0% CMM and 50 mM sodium acetate buffer (pH 6.0), and incubated at 15°C for 24 h. Samples were spotted on a TLC plate, developed, and then detected by 20% sulfuric acid. Lane M, glucose and maltooligosaccharides standard solution (G1, glucose; G2, maltose; and so on). Lane 1, CMM standard; lane 2, α-maltosyl-(1→6)-maltose standard; lane 3, cell-free extract of *A. globiformis* M6 without CMM; lane 4, after the reaction with cell-free extract from BL21 (DE3) transformed with pRSETA; lane 5, after the reaction with cell-free extract from BL21 (DE3) transformed with pRSET-CMMase. Saccharide 1 on lane 5 indicates α-maltosyl-(1→6)-maltose.

When cell extract solution prepared from *E. coli* BL21 (DE3) harboring the original expression vector pRSETA was incubated with CMM, the production of α-maltosyl-(1→6)-maltose was not observed (Fig. 4, lane 4). Based on these results, we concluded that the isolated gene actually encodes CMMase. The CMM hydrolyzing activity of the cell extract of *E. coli* BL21 (DE3) harboring pRSET-CMMase was 0.028 U/mL. CMM was not digested completely by the enzyme solution because of the low enzymatic activity and a low reaction temperature (lane 5). No CMM hydrolyzing activity was detected from the cell extract of *E. coli* BL21 (DE3) harboring pRSETA.

Homology searches revealed that the deduced amino-acid
sequences of CMMase (amino-acid positions 1 to 450) showed similarities to GH 13 or α-amylase family enzymes,11, 12 and 46, 46 and 45% identities to CDase from *Geobacillus kaustophilus*, maltogenic α-amylase from *Bacillus* sp. and neopullulanase from *Bacillus stearothermophilus*,19,21 respectively (Fig. 5). The four conserved regions that are common in this family of enzymes were also found in CMMase, indicating that this enzyme should be assigned to this family. CMMase has a characteristic amino-acid sequence within conserved region II, namely Pro203-Tyr204-Phe205. Proline, tyrosine or phenylalanine has a cyclic aliphatic or an aromatic side chain in its structure, and is hydrophobic. Therefore, we expected the structure in the vicinity of the substrate binding region of CMMase which was different from CDase, maltogenic α-amylase, neopullulanase and Pro203-Tyr204-Phe205 to limit the direction of the substrate. Because the direction of the substrate was limited, the substrate which efficiently bound to the substrate binding region of CMMase was expected to be limited to CMM. Crystal and mutational analyses of CMMase should provide insight into the relationship between the structure and substrate specificity.

**Other ORFs.**

The ORF-2 (*cmmC*) gene was located just downstream of *cmmB* through a 53 bp flanking region. The structural gene was expected to encode a protein with 421 amino acid residues (calculated molecular mass, 44,547 Da). The ORF started at the ATG initiation codon (nucleotide position 661) and ended at the TGA stop codon at position 1,924. A putative SD, AGGAGA, preceded the ATG initiation codon. Homology searches using a non-redundant protein sequence database revealed that the deduced amino-acid sequences of CmmC showed 27% identities to the sugar-binding protein of the ABC-type sugar transport system from *Streptomyces avermitilis*.22

The ORF-3 (*cmmD*) gene lies in the rear of ORF-2 through a short flanking region of 10 bp. This gene extends from the ATG initiation codon (nucleotide position 1,937) to the TGA stop codon at position 2,897. A putative SD, GAGCGG (nucleotide positions 1,925 to 1,930), preceded the ATG initiation codon. ORF-3 was expected to encode a protein with 320 amino acid residues (calculated molecular mass, 34,651 Da). Homology searches using a non-redundant protein sequence database revealed that the deduced amino-acid sequences of ORF-3 showed 44% identities to the putative permease of the ABC-type sugar transport system from *Deinococcus geothermalis*.22

The ORF-3 (*cmmD*) gene lies in the rear of ORF-2 through a short flanking region of 10 bp. This gene extends from the ATG initiation codon (nucleotide position 1,937) to the TGA stop codon at position 2,897. A putative SD, GAGCGG (nucleotide positions 1,925 to 1,930), preceded the ATG initiation codon. ORF-3 was expected to encode a protein with 320 amino acid residues (calculated molecular mass, 34,651 Da). Homology searches using a non-redundant protein sequence database revealed that the deduced amino-acid sequences of ORF-3 showed 44% identities to the putative permease of the ABC-type sugar transport system from *Deinococcus geothermalis*.22

The ORF-4 (*cmmE*) encodes a protein of 284 amino acid residues (calculated molecular mass of 31,764 Da). The ORF
starts from the ATG initiation codon at position 2,863 preceded by a potential SD, GAGTGA and ends at the TGA stop codon at position 3,715. This gene was overlapping with the 37 bp region of ORF-3. The deduced amino-acid sequences showed 49% identities to the putative permease of the ABC-type sugar transport system from *Bacillus clausii*. Described above, CmmD was similar to the possible permease from *D. geothermals*. The identities suggest that the proteins encoded by ORF-3 and ORF-4 form a subunit structure and function as permease.

ORF-6 (cmmG), which lies rear of cmmF through a short flanking region of 17 bases, is an incomplete ORF, of which 3'-region is not sequenced yet. The ORF starts from the ATG initiation codon at position 5,094 preceded by a potential SD, GACG GG. The amino-acid sequence (194 residues) deduced from the ORF shows similarity to the N-terminal region of a transcriptional regulator from *Glucobacter oxydans* (35% identity).21

No promoter or transcriptional terminator was found between cmmB and cmmG. Hence we considered that cmmB, cmmC, cmmD, cmmE, cmmF and cmmG might form a gene cluster together with cmmA (6MT gene), namely cmmABCDEFG. In the case of CDs, the cgtBACDE cluster was found in the genome of *Thermococcus* sp. B1001: cgtB for the intracellular CDase, cgtA for the extracellular CGTase, cgtC for the CD-binding protein, and cgtDE for the membrane transporter proteins. 13) It has been reported that *Klebsiella oxytoca* M5a1 has a CD-synthesis/uptake/degradation system similar to that of *Thermococcus*.24 This system is considered to be advantageous for these microorganisms assimilating starch as a carbon source, because CDs synthesized from starch by the action of CGTase show various degrees of resistance and inhibition to amylolytic enzymes produced by competitive microorganisms. Additionally, the competitors having the usual transport system for linear maltooligosaccharides are unable to take CDs into cells. We suppose that CMM has a similar advantage to CDs. For the purpose of revealing the metabolic pathway of CMM, functional analysis of CmmC, CmmD and CmmE is in progress.

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