Paenibacillus sp. 598K 6-α-glucosyltransferase is essential for cycloisomaltooligosaccharide synthesis from α-(1→4)-glucan

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--Manuscript Draft--

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Abstract:	Paenibacillus sp. 598K produces cycloisomaltooligosaccharides (cyclodextrans) from starch, even in the absence of dextran. Cycloisomaltooligosaccharide glucanotransferase synthesizes cycloisomaltooligosaccharides exclusively from an α-(1→6)-consecutive glucose chain consisting of at least four molecules. Starch is not a substrate of this enzyme. Therefore, we predicted that the bacterium possesses another enzyme system for extending α-(1→6)-linked glucoses from starch, which can be used as the substrate for cycloisomaltooligosaccharide glucanotransferase, and identified the transglucosylation enzyme Ps6GT31A. We purified Ps6GT31A from the bacterial culture supernatant, cloned its corresponding gene, and characterized the recombinant enzyme. Ps6GT31A belongs to glycoside hydrolase family 31, and it liberates glucose from the non-reducing end of the substrate in the following order of activity: α-(1→4)- > α-(1→2)- > α-(1→3)- > α-(1→6)-glucobiose and maltopentaose > maltotetraose > maltotriose > maltose. Ps6GT31A catalyzes both hydrolysis and transglucosylation. The resulting transglucosylation compounds were analyzed by high-performance liquid chromatography and mass spectrometry. Analysis of the initial products by 13C nuclear magnetic resonance spectroscopy revealed that Ps6GT31A had a strong α-(1→4) to α-(1→6) transglucosylation activity. Ps6GT31A elongated α-

	(1→6)-linked glucooligosaccharide to at least a degree of polymerization of 10 through a successive transglucosylation reaction. Eventually, cycloisomaltooligosaccharide glucanotransferase creates cycloisomaltooligosaccharides using the transglucosylation products generated by Ps6GT31A as the substrates. Our data suggest that Ps6GT31A is the key enzyme to synthesize α-(1→6)-glucan for cycloisomaltooligosaccharide production in dextran-free environments.
Response to Reviewers:	See attachment.

To
International Editor
Applied Microbiology and Biotechnology

Dear Dr. Kunihiko Watanabe

Thank you for the decision letter concerning about our manuscript entitled "*Paenibacillus* sp. 598K 6-α-glucosyltransferase is essential for cycloisomaltooligosaccharide synthesis from α-(1→4)-glucan" (Ref.: Ms. No. AMAB-D-16-02995). In accordance with the reviewer's comments, we revised our paper. We corrected some words and sentences in Abstract and text, according to the comments of Reviewer #1. Also, we have put some sentences in the text or figure legend for explaining more about Figures 2 and 7. The marked and unmarked revised manuscripts have been submitted. We hope that you will consider this revised manuscript suitable for publication in Applied Microbiology and Biotechnology.

Sincerely yours,
Kazumi Funane, Ph. D.
Food Research Institute, National Agriculture and Food Research Organization (NARO)

Responses to Reviewers

To
Reviewer #1

Reviewers' comments:

Reviewer #1: Please enter your comments to the Author below:
Ichinose and colleagues have isolated a protein from Paenibacillus that produces cyclic dextrans, and, after N-terminal sequencing, isolated the gene, cloned it and produced the protein recombinantly. Characterization of the protein showed that it has both hydrolytic and glucotransferase activity, producing cyclodextrans containing 8-10 glucose residues from starch.

The work is described in very good detail, but in a somewhat inaccessible way. For instance, see my comments for lines 272-274 and 276-278. I had also difficulty in understanding Figure 7b by just reading the main text; reading the legend contains experimental information that is absolutely necessary to understand the figure.

Technically, the work has been done and described to my satisfaction.

Below I give a few suggestions for imrpovement.

Thank you for reviewing our manuscript and thank you very much for your positive and useful comments. As suggested, we revised the manuscript.

Line 28: The starch is not the substrate -> Starch is not a substrate Line 29: possessed -> possesses Line 36: catalyzed -> catalyzes; resultant -> resulting Line 38: The analysis -> Analysis Line 40: by at least -> to at least Line 45: survival: are cycloisomaltooligosaccharides essential for survival of the bacterium? Please, provide data or a reference.

Response
As suggested, we have correct the words in the abstract at Lines 28, 29, 36, 37, 38, and 40 in the marked manuscript and Lines 28, 29, 36, 38, and 40 in the unmarked manuscript.
At Line 45, ‘to enable survival’ has been removed because the word “survival” may give readers false information. Even if the bacteria use CI for nutrient source, giving the impression that CI is

essential for the bacteria would be going too far. Thank you for your kind suggestion. We revised the sentence as follows.

Lines 272-274: it is not clear to this reviewer why adding native CITase to fractions 38-45 increases the starch transformation activity. Please, explain.

Response

CITase catalyzes CI-production from α-(1,6)-glucose chains but it does not have the α-(1,4) to α-(1,6) transglucosylation activity. So CITase have to rely on another enzyme which produce α-(1,6)-glucose chains from α-(1,4)-linked glucose chains when it produces CIs from starch. We tried to find the enzyme (6GT31A) which produce α-(1,6)-glucose chains from starch in the bacterial culture supernatants. The fractions 38-45 contain 6GT31A and a small amount of CITase (Fig. 2). When fraction 49 (native CITase) was added to these fractions, amount of CITase was increased, and CI-production from starch was also increased. We think large amounts of α-(1,6)-glucose chains are able to be produced from starch in these fractions. And the peak fraction 41 can produce enough amounts of α-(1,6)-glucose chains to express the maximum CI-producing activity of the CITase in fraction 41+additional fraction 49. We have removed the explanation about fraction 45 from the original paper, and instead, we add the explanation about the peak fraction 41 to make the point clearer. We revised the sentences as follows.

Lines 262-266 in the marked revised manuscript and Lines 262-264 in the unmarked revised one: CITase produces CIs from α-(1→6)-consecutive glucose chains of DPs≥4. To make CIs from starch, the bacterium would need to possess the enzyme for elongating α-(1→6)-linkages from starch. We therefore attempted to purify this enzyme.

Lines 275-284 in the marked revised manuscript and Lines 273-277 in the unmarked revised one: Among them, the peak fraction 41 reached its CI-producing activity against starch to about 2.4 U/mL. That is approximately the same as the sum of the CI-producing activity against dextran 40 of the shoulder peak and fraction 49 (Fig. 2, open circles). The enzyme eluted at around fraction 41 must produce α-(1→6)-glucose chains from starch usable for CITase.

Lines 276-278: this is a complicated sentence (5-60 words!) with too many "fractions". Please, make shorter sentences, and clarify the point that you want to make. (Part of the problem is probably that I do not understand the purpose of fraction 49 in lines 272-274.

Response

As suggested, we revised the sentence as follows. We cut the original sentence into 4 sentences to make one sentence shorter as follows.

Lines 284-294 in the marked revised manuscript and Lines 277-282 in the unmarked revised one: The rest of the fractions except fractions 38–45 shown in Fig. 2 observed no CITase activity against starch even with additional CITase. The Resource Q column after 0–600 mM NaCl graidient elution was washed further with 1 M NaCl. The eluted proteins did not show any CI-producing activity against starch. Also, the precipitated proteins of 598K culture supernatants with <20% and >60% ammonium sulfate-saturated showed no CITase activity against starch.

Line 44); produce -> produces

Response

It may be the line 440 in the original manuscript. As suggested, we revised as follows.

Line 456 in the marked revised manuscript and Line 444 in the unmarked revised one:
…. enzyme that produces a substrate for CITase to synthesize CIs using starch or …..

Figure 7b: this is not clear: if fraction 7 is incubated without enzyme, nothing should happen, and a peak corresponding to G7 should be visible. This is not the case. Why not? Perhaps, the authors should explain Figure 7c before showing 7b?

Response

Figure 7b shows the results of HPLC analysis after glucoamylase (/α-1,4/α-1,6) and highly branched dextran hydrolase (/α-1,4/α-1,6/α-1,3/α-1,2)-digestion of the reaction mixture. When fraction 7 was incubated without CITase, heptasaccharides were remained in the reaction mixture. But the heptasaccharides were hydrolyzed to glucose by subsequent enzyme digestion. We added a sentence in the results section and revised the figure legend as follows.

Lines 409-411 in the marked revised manuscript and Lines 397-399 in the unmarked revised one: Then the reaction mixture was incubated with glucoamylase and highly branched dextran hydrolase. The remaining linear oligosaccharides are digested completely to glucose with this

treatment, and intact cyclic oligosaccharides are left.

Line 720-723 in the marked revised manuscript and Lines 706-708 in the unmarked revised one: Fig.7…. (b) …..The reaction products were digested with glucoamylase and highly branched dextran hydrolase, and they were analyzed by HPLC-ELSD as described previously (Funane et al. 2014).

To
Reviewer #2

Reviewer #2: Please enter your comments to the Author below:

This is extraordinarily good scientific paper. The study fills important gaps in understanding the mechanisms of synthesis of cyclodextrans. The experiments are meaningfully done. Results are reliable throughout. Conclusions are justified. Presentation does not require editing.

Thank you for reviewing our manuscript and thank you very much for giving it high evaluation. We made some revision for the manuscript in accordance with the other reviewer's comments.

Paenibacillus sp. 598K 6-α-glucosyltransferase is essential for cycloisomaltooligosaccharide synthesis from α-(1→4)-glucan

Hitomi Ichinose[1,5,6], Ryuichiro Suzuki[1,7],Takatsugu Miyazaki[1,8],Keitarou Kimura[1], Mitsuru Momma[2], Nobuhiro Suzuki[3], Zui Fujimoto[2], Atsuo Kimura[4], Kazumi Funane[1,*]

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Abstract

Paenibacillus sp. 598K produces cycloisomaltooligosaccharides (cyclodextrans) from starch, even in the absence of dextran. Cycloisomaltooligosaccharide glucanotransferase synthesizes cycloisomaltooligosaccharides exclusively from an α-(1→6)-consecutive glucose chain consisting of at least four molecules. ~~The s~~Starch is not a~~the~~ substrate of this enzyme. Therefore, we predicted that the bacterium possesse~~d~~ another enzyme system for extending α-(1→6)-linked glucoses from starch, which can be used as the substrate for cycloisomaltooligosaccharide glucanotransferase, and identified the transglucosylation enzyme Ps6GT31A. We purified Ps6GT31A from the bacterial culture supernatant, cloned its corresponding gene, and characterized the recombinant enzyme. Ps6GT31A belongs to glycoside hydrolase family 31, and it liberates glucose from the non-reducing end of the substrate in the following order of activity: α-(1→4)- > α-(1→2)- > α-(1→3)- > α-(1→6)-glucobiose and maltopentaose > maltotetraose > maltotriose > maltose. Ps6GT31A catalyze~~d~~ both hydrolysis and transglucosylation. The resulting~~ant~~ transglucosylation compounds were analyzed by high-performance liquid chromatography and mass spectrometry. ~~The a~~Analysis of the initial products by ^{13}C nuclear magnetic resonance spectroscopy revealed that Ps6GT31A had a strong α-(1→4) to α-(1→6) transglucosylation activity. Ps6GT31A elongated α-(1→6)-linked glucooligosaccharide to~~by~~ at least a degree of polymerization of 10 through a successive transglucosylation reaction. Eventually, cycloisomaltooligosaccharide glucanotransferase creates cycloisomaltooligosaccharides using the transglucosylation products generated by Ps6GT31A as the substrates. Our data suggest that Ps6GT31A is the key enzyme to synthesize α-(1→6)-glucan for cycloisomaltooligosaccharide production ~~to enable survival~~ in dextran-free environments.

Keywords: cycloisomaltooligosaccharide, 6-α-glucosyltransferase, glycoside hydrolase family 31, *Paenibacillus* sp. 598K, starch

Introduction

Cycloisomaltooligosaccharide (cyclodextran, CI) is a bacterial cyclic oligosaccharide consisting of α-(1→6)-linked glucosyl residues (all of the sugars in the present study are in the D-configuration unless otherwise specified) (Funane et al. 2008; Oguma et al. 1993). CI is highly hydrophilic, and it is an anti-plaque carbohydrate with strong inhibitory activity against streptococcal glucansucrases (Kobayashi et al. 1995). CI forms an inclusion complex with insoluble and/or unstable compounds; that is, CI-7 and CI-8 (CI-*n*, where *n* is the number of glucose molecules) solubilize C_{60} and C_{70} fullerenes (Jina et al. 1996), and CI-10 stabilizes Victoria blue B (Funane et al. 2007).

To date, three CI-producing bacteria, *Paenibacillus agaridevorans* T-3040 {formerly *Bacillus circulans* T-3040 [FERMBP-4132 (NBRC)]; 16S rRNA accession number, LC042199, K. Ochi, personal communication}, *Paenibacillus* sp. 598K, and *B. circulans* U-155, have been reported (Funane et al. 2008; Oguma et al. 1993, 2014). When the bacteria are grown in the presence of dextran, expression of the extracellular enzyme cycloisomaltooligosaccharide glucanotransferase (CITase; EC 2.4.1.248) is induced and CIs are simultaneously produced from dextran by CITase in the culture supernatants (Oguma et al. 1994, 2014; Suzuki et al. 2012). CITase catalyzes the intramolecular (cyclization) and intermolecular (disproportionation, coupling) transglycosylation and hydrolysis reactions of α-(1→6)-glucan (dextran) (Oguma et al. 1994) and isomaltooligosaccharides [degree of polymerization (DP) ≥4] (Suzuki et al. 2012).

Dextran is one of the exopolysaccharides produced by lactic acid bacteria such as *Leuconostoc*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, and *Weissella* (Monsan et al. 2001; Torino et al. 2015), as well as *Rhizopus* spp. (Samkpal et al. 2001). They produce extracellular dextransucrase (EC 2.4.1.5) that synthesizes dextran from sucrose (Leemhuis et al. 2013). Another dextran-producing bacterium, *Gluconobacter oxydans*, produces dextran dextrinases (EC 2.4.1.2) that synthesize dextran from maltodextrins (Naessems et al. 2005). As no reports of dextran synthase in CI-producing bacteria have been published, there were two possibilities: CI-producing bacteria synthesize dextran using an unknown enzyme system by themselves, or CI-producing bacteria utilize dextran produced by other dextran-producing bacteria. Recently, we reported that *P. agaridevorans* T-3040 produces CI from starch even in the absence of dextran (Funane et al. 2014). The bacterium autonomously produces CI from starch, and a 135-kDa protein possessing transglucosylation activity with maltooligosaccharides was assumed to be required for CI production from starch, although the details remain unclear.

In this study, *Paenibacillus* sp. 598K was cultured in a medium containing various carbon sources, and CI-producing activity was investigated to determine whether the bacterium possesses

an enzyme system for CI production without dextran. We found a key enzyme for α-(1→6)-glucan synthesis from starch and named it Ps6GT31A. It belongs to glycoside hydrolase family 31 (GH31), which consists of diverse enzymes such as α-glucosidase (EC 3.2.1.20), α-xylosidase (EC 3.2.1.177), and oligosaccharide α-1,4-glucosyltransferase (EC 2.4.1.161). In this study, we report the detailed characteristics of Ps6GT31A and discuss its involvement in CI production from starch.

Materials and Methods

Substrates

Pregelatinized starch (Matsunorin M-22 starch) was purchased from Matsutani Chemical Industry Co., Ltd. (Itami, Japan). Dextrin was obtained from Becton, Dickinson and Company (Sparks, USA), and dextran 40 was acquired from GE Healthcare, UK, Ltd. (Little Chalfont, UK). Maltose (G2), maltotriose (G3), pullulan, which commonly consists of α-(1→6) linked maltotriose, kojibiose, nigerose, and cellobiose were procured from Wako Pure Chemical Industries (Osaka, Japan). Maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), maltoheptaose (G7), and Fujioligo G67 (maltohexaose- and maltoheptaose-rich maltooligosaccharides) were purchased from Nihon Shokuhin Kako, Co., Ltd. (Tokyo, Japan). Isomaltooligosaccharides (IG2–IG7; of which the number represents glucose molecules) were acquired from Seikagaku Co. (Tokyo, Japan). Isomalto 500 (a mixture of IG2, IG3, and panose) was obtained from Showa Sangyo Co., Ltd. (Tokyo, Japan). Trehalose was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and gentiobiose, sophorose, panose, *p*-nitrophenyl (PNP) α-glucopyranoside, PNP α-mannopyranoside PNP α-galactopyranoside, and PNP α-xylopyranoside were procured from Sigma Chemical Company (St. Louis, MO, USA). Laminaribiose was obtained from Megazyme International (Bray, Ireland). Standard CIs (CI-7–CI-9) were acquired from C-I Bio Ltd. (Tomigusuku, Japan). Glucans from *Leuconostoc mesenteroides* NRRL B-1299 [B1299 glucan, which contains α-(1→6) and α-(1→2) linkages] (Brison et al. 2012) and NRRL B-1355 [B1355 glucan, which contains α-(1→6) and α-(1→3) linkages] (Côte and Robyt 1982) were kindly donated by Dr. M. Kobayashi.

CI-producing activity against dextran and starch in the culture supernatants of a medium containing various carbon sources

Paenibacillus sp. 598K [FERM P-19604(NBRC)] was cultured in 2 mL of Luria–Bertani (LB) broth, which was supplemented with or without 2% (w/v) carbohydrate sources, namely, glucose, sucrose, Fujioligo G67, dextrin, Matsunorin M-22 starch, Isomalto 500, nigerose, kojibiose, pullulan, B1299 glucan, B1355 glucan, dextran 40, or 2% (w/v) dextran 40 with 1% (w/v) glucose at 30°C for 3 days with shaking at 200 min^{-1}. Each of the culture broths was centrifuged at 10,000 × *g* for 10 min, and the supernatants were collected. Each culture supernatant was desalted and concentrated using an Amicon Ultra filter (10,000 MWCO; Merck Millipore, Billerica, MA, USA), and CI-producing activity against dextran and starch was determined as described previously (Funane et al. 2014). Briefly, the sums of the amounts of CI-7, CI-8, and CI-9 were quantified by high-performance liquid chromatography with an evaporative light scattering detection system (HPLC-ELSD; LC Workstation Class-VP;

Shimadzu, Co., Kyoto, Japan) using a TSK gel Amide-80 column (4.6 × 250 mm; Tosoh, Co., Tokyo, Japan). The experiments were performed in triplicate. One unit of CITase activity was defined as the amount of enzyme that released 1 μmol of the sum of CI-7, CI-8, and CI-9 per minute.

Purification and sequence analysis of native Ps6GT31A

Paenibacillus sp. 598K was grown in 400 mL of LB medium containing 2% (w/v) dextran 40 in a baffled flask at 30°C for 3 days with shaking at 160 min^{-1}. The culture was centrifuged at 10,000 × *g* for 10 min. Ps6GT31A was purified from the supernatant by precipitation with 20%–60% saturated ammonium sulfate followed by Resource Q chromatography (GE Healthcare) twice. The proteins were dialyzed against 20 mM Tris-HCl (pH 8.0). The bound proteins were eluted using a 0–600 mM NaCl linear gradient. The CITase activity against dextran 40 and starch in each fraction was measured as described above. Purified protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a PVDF membrane. The sequence of the 23 N-terminal amino acids was determined using an HP G1005A protein sequencer (Hewlett-Packard, Palo Alto, CA, USA).

Because the DNA fragment encoding the N-terminal amino acid sequence was found in the 5269-bp *Hin*dIII DNA fragment (GenBank accession no. DJ083453), which contained the full-length *cit* gene (GenBank accession no. AB685169) reported previously (Suzuki et al. 2012), the *Hin*dIII DNA fragment was used as a probe for selecting the Ps6GT31A-encoding gene *6gt31a* from genomic DNA libraries. The resultant 7519-bp *Hin*dIII–*Nde*I DNA fragment containing full-length *cit* and *6gt31a* and partial hypothetical protein-coding genes were sequenced.

Expression and purification of recombinant Ps6GT31A

The gene encoding mature Ps6GT31A (Ala36–Pro1281) without a secretion signal sequence (Met1–Ala35) was amplified from the genomic DNA by PCR using the following primers: forward, 5′-CATATGGCCGGGCTCGGCAATG-3′; and reverse, 5′-GGATCCTTAAGGCGCTCGGGTGAG-3′. The amplified DNA was cloned into pET15b vectors (Novagen, Inc., Madison, WI, USA) at *Nde*I and *Hin*dIII restriction enzyme sites (underlined). *Escherichia coli* BL21 (DE3) cells (Novagen) harboring the expression plasmid were cultured, and the expression of N-terminal fusion His-tagged Ps6GT31A was induced with 0.1 mM isopropylβ-thiogalactopyranoside for 24 h at 16°C. The cells were harvested and resuspended in 50 mM potassium phosphate-NaOH buffer (pH 7.2), followed by sonication for 5 min. After centrifugation to remove insoluble material, the supernatant was loaded onto a 1-mL HisTrap HP column (GE Healthcare) equilibrated with the same buffer. The bound enzyme was eluted with 50 mM potassium phosphate-NaOH buffer (pH 7.2) containing 250 mM imidazole. The eluted

enzyme, identified by SDS-PAGE as a 136-kDa protein, was dialyzed against 20 mM Tris-HCl (pH 8.0). It was further purified by being loaded onto a Mono Q column (GE Healthcare) equilibrated with the same buffer and eluted with a linear gradient of 0–1 M NaCl in 20 mM Tris-HCl (pH 8.0). Two major peaks of Ps6GT31A were obtained. The peak eluted at the lower salt concentration was the monomer and the one eluted at the higher salt concentration was the dimer determined by gel filtration chromatography using a Superose 12 column (GE Healthcare). The former fraction was dialyzed against 20 mM Tris-HCl (pH 8.0) and used as the purified enzyme. The protein concentration was determined by measuring absorbance at 280 nm, assuming that an absorbance value of 1.0 indicated a concentration of 0.48 mg/mL (molar extinction coefficient = 290,270 $M^{-1} \cdot cm^{-1}$).

Detection of mono- and oligosaccharides

Hydrolytic and transglucosylation products generated by Ps6GT31A were detected by thin-layer chromatography (TLC; TLC Silica gel 60 F_{254} plates; Merck Millipore) with an appropriate solvent or by HPLC-ELSD with a TSK gel Amide-80 column (4.6 × 250 mm) as described above.

α-Glucosidase activity of Ps6GT31A

The reaction mixture consisted of 300 μL of 0.1 M Tris-malate buffer (pH 6.0), 500 μL of 1% (w/v) starch, and 100 μL of 0.002% (w/v) L-rhamnose (internal standard). After pre-incubation at 50°C for 10 min, 100 μL of the enzyme preparation was added, and the reactions were performed at 50°C. At regular time intervals, 100-μL aliquots of the reaction mixture were obtained. After heat incubation at 100°C for 5 min, α-glucosidase activity was determined by the released glucose quantified by HPLC-ELSD using the same column as above with an acetonitrile–water (60:40, v/v) mixture as the mobile phase at 30°C and a flow rate of 1 mL/min. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol glucose per minute from the substrate under these conditions. The substrate specificity of Ps6GT31A for polysaccharides was also determined by the amounts of glucose released.

The effect of temperature on the enzyme activity was examined at a set temperature instead of 50°C for 10 min. Concerning the effect of temperature on enzyme stability, the enzyme (12 nM) dissolved in 20 mM Tris-HCl buffer (pH 8.0) was incubated at a set temperature for 1 h. Then, it was used for the enzyme assay at 50°C for 10 min. The effects of pH on enzyme activity were examined using 50 mM sodium acetate buffer (pH 4.0–5.5) and 0.1 M Tris-malate buffer (pH 5.5–8.0), as well as Atkins–Pantin buffer (0.2 M boric acid/0.2 M KCl/0.2 M Na_2CO_3, pH 8.0–11.0) substituted for 0.1 M Tris-malate buffer (pH 6.0). Regarding the effects of pH stability on enzyme activity, the enzyme was incubated at 37°C for 1 h, and then it was used for the enzyme assay as described above.

The substrate specificity of the enzyme for glucobioses was analyzed using G2 [α-Glc-(1→4)-Glc], kojibiose [α-Glc-(1→2)-Glc], nigerose [α-Glc-(1→3)-Glc], IG2 [α-Glc-(1→6)-Glc], trehalose [α-Glc-(1↔1)-α-Glc], cellobiose [β-Glc-(1→4)-Glc], sophorose [β-Glc-(1→2)-Glc], laminaribiose [β-Glc-(1→3)-Glc], and gentiobiose [β-Glc-(1→6)-Glc]. Briefly, an aliquot of enzyme (0.31–2.0 μM) was incubated with 100 μM substrate in 30 mM Tris-malate buffer (pH 6.0) for up to 120 min at 50°C. The amount of each product was quantified by HPLC-ELSD. To assess the catalytic efficiency of the enzyme for maltooligosaccharides, the enzyme (4–312 nM) was incubated with 100 μM substrate. Progress curves of oligosaccharide cleavage were used to determine k_{cat}/K_m. The activity for PNP glycosides was determined as follows. The reactions were performed in 30 mM Tris-malate buffer (pH 6.0) containing 1–5 mM substrates and 0.7 μM enzyme at 37°C. The amount of *p*-nitrophenol released was determined from the absorbance at 400 nm (molar extinction coefficient = 2,213 $M^{-1}\cdot cm^{-1}$). The assay was performed in triplicate. The kinetic parameters k_{cat} and K_m were determined using Eadie–Hofstee plots.

Transglucosylation activity of Ps6GT31A

The transglucosylation/hydrolysis ratios for maltooligosaccharides were determined as follows. The reactions were performed in 30 mM Tris-malate buffer (pH 6.0) containing 0.4 mM substrates and 15 nM–1.4 μM enzyme at 50°C for up to 20 min. The amounts of substrate (G_n, where n is the number of glucose molecules), hydrolysis products (glucose and G_{n-1}), and transglucosylation products (G_{n+1}) were quantified by HPLC-ELSD. To analyze the reaction products of G4 and IG4 at the initial stage, the enzyme (60 nM for G4, 0.8 μM for IG4) was incubated with the substrate (0.8 mM G4 or 1.7 mM IG4) in 30 mM Tris-malate buffer (pH 6.0) at 50°C.

Reaction products of G4 at the initial stage were purified by HPLC with a refractive index detector (RID-10A; Shimadzu) and a TSKgel Amide-80 column (21.5 × 300 mm; Tosoh) using an acetonitrile–water (55:45, v/v) mixture as the mobile phase at 30°C and a solvent flow rate of 5 mL/min. The lyophilized products were analyzed by Fourier transform mass spectrometry (FT-MS) and ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy. The mass spectra were determined using a JMS HX-100/100A mass spectrometer (JEOL, Ltd., Tokyo, Japan) via fast-atom bombardment ionization. The ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin, Karlsruhe, Germany) at 298K.

Enzymatic treatment of reaction products generated by Ps6GT31A

Reaction products were generated by Ps6GT31A (35 nM) reacting with 1% (w/v) G5 and 0.1% (w/v) bovine serum albumin in water at 37°C for 24 h and separated into 10 fractions by HPLC (fractions 1–10). Nine fractions (fractions 2–10) were analyzed by FT-MS. Each product

(0.1 mg) was incubated with oligo-1,6-glucosidase (Megazyme, 10 units) in 20 mM sodium acetate buffer (pH 4.5) or a mixture of oligo-1,6-glucosidase (5 units) and α-glucosidase from *Bacillus stearothermophilus* (Megazyme, 5 units) in 20 mM Tris-malate buffer (pH 6.0) at 40°C for 20 h. The hydrolytic products were analyzed by HPLC-ELSD. The assay was performed in duplicate.

CI production by CITase from transglucosylation products generated by Ps6GT31A

Fractions 2–10 (5 mM) mentioned above were individually incubated with 100 nM recombinant *Paenibacillus* sp. 598KCITase (Suzuki et al. 2012) in 30 mM Tris-malate buffer (pH 6.0) at 37°C for 24 h and boiled at 100°C for 10 min. CI-producing activity was measured as the total production of CI-7, CI-8, and CI-9 after enzymatic digestion of the remaining linear glucooligosaccharides with highly branched dextran hydrolase and glucoamylase, as described previously (Funane et al. 2014; Suzuki et al. 2012).

GenBank accession numbers

The partial sequence of the 16S rRNA gene of *Paenibacillus* sp. 598K has been deposited with GenBank under the accession number LC155798. The *Hin*dIII-*Nde*I nucleotide sequence of the 7519-bp segment containing full-length *cit* and *6gt31a* genes and partial hypothetical protein-coding gene was deposited in GenBank/DDBJ under the accession number LC160266.

Results

Identification of native Ps6GT31A

Paenibacillus sp. 598K was cultivated in a medium containing various carbon sources, namely glucose, sucrose, Fujioligo G67, dextrin, Matsunorin M-22 starch, Isomalto 500, pullulan, nigerose, kojibiose, B1299 glucan, B1355 glucan, and dextran 40 (Figure S1), and the CI-producing activity of the secreted enzyme in the culture supernatant against dextran or starch was examined by HPLC, as described previously (Fig. 1) (Funane et al. 2014). At least a trace amount of the CI-producing activity against both dextran and starch was observed in all cultures, and when the bacterium was grown with pullulan, B1299 glucan, B1355 glucan, and dextran 40, the activity clearly increased. The level of CI-producing activity of the pullulan culture was much lower than that of the others. Its growth [optical density (OD) at 600 nm] reached only 0.35 after 3-day cultivation, whereas those of B1299 glucan, B1355 glucan, and dextran 40 cultures reached 1.5–2.0. With other carbohydrates, no significant CI-producing activity was observed (Fig. 1). When the carbon source was dextrin, Matsunorin M-22 starch, or Isomalto 500, bacteria did not grow well at the beginning, the lag phase of which was considerably long (22–35 hours) (Figure S1). CI-producing activities against both dextran and starch were unaffected by glucose addition in dextran 40-supplemented culture broth.

CITase produces CIs from α-(1→6)-consecutive glucose chains of DPs≥4. To make CIs from starch, the bacterium would need to possess the enzyme for elongating α-(1→6)-linkages from starch. ~~Because CIs were produced from starch without dextran, but CITase itself could not produce CIs from starch, the bacterium was believed to possess an enzyme system for converting starch to dextran.~~ We therefore attempted to purify ~~the~~ this enzyme. When the crude enzymes, which were pre-purified by ammonium sulfate precipitation from the culture supernatant of the medium containing dextran 40, were subjected to anion chromatography to measure CITase activity against starch (Fig. 2), low CI-producing activity was measured in fractions 38–45 (Fig. 2, solid squares). In contrast, as indicated by the open circles in Fig. 2, high-CI-producing activity against dextran was observed in fractions 45–58, and a shouldered peak was also observed in fractions 38–45, which is comparable to the CI-producing activity against starch. Fractions 45–58 were considered to be CITase because CIs were produced from dextran 40 but not from starch with these fractions. Then, native CITase (fraction 49) was added to the fractions, and CITase activity against starch was remarkably increased at fraction 38-44 (Fig. 2, solid triangles). Among them, the peak fraction 41 reached its CI-producing activity against starch to about 2.4 U/mL. That is approximately the same as the sum of the CI-producing activity against dextran 40 of the shoulder peak and fraction 49 (Fig. 2, open circles). ~~CITase catalyzes disproportionation, coupling~~

and cyclization of isomaltooligosaccharides. Accumulation of long isomaltooligosaccharides results in the large amount of CI production. Fraction 45, which showed some CITase activity against both starch and dextran 40, but did not show significantly increased CITase activity against starch with additional CITase. Probably, smost probably because sufficient amounts of CITase had already been included in this fraction 45. The enzyme eluted at around fraction 41 must produce α-(1→6)-glucose chains from starch usable for CITase. The rest of the fractions except fractions 38–45 shown in Fig. 2 observed no CITase activity against starch even with additional CITase. BothThe remaining proteins eluted with 1 M NaClResource Q column –after 0–600 mM NaCl graidient elution was washed further with 1 M NaCl. of the first Resource Q column chromatography, The eluted proteins did not show any CI-producing activity against starch. andAlso, the precipitated proteins of 598K culture supernatants with <20% and >60% amnnmonium sulfate-saturated also showed no CITase activity against starch. In other fractions, that is, <20% and >60% ammonium sulfate saturated fractions of 598K culture supernatants, other fractions besides fractions 38–45 shown in Fig. 2, and remaining proteins eluted with 1 M NaCl after 0–600 mM NaCl fractionation of the first Resource Q column chromatography, no CITase activity against starch was observed, even when Fraction 49 CITase was added. Therefore, we assumed that the protein eluted at fractions 38–45 was the only enzyme involved in extracellular CI production from starch with CITase in *Paenibacillus* sp. 598K. Fractions 38–45 were thus further purified into a single 135-kDa band on SDS-PAGE (Figure S2, lane 4). The protein in this band exhibited both hydrolytic and transglucosylation activity against maltooligosaccharides, similarly to the 135-kDa protein from *P. agaridevorans* T-3040 (Funane et al. 2014), and it was named Ps6GT31A. Edman analysis of the purified native Ps6GT31A yielded an N-terminal amino acid sequence of AGLGNVTGAVASGDSLTLTLDNG. From the N-terminal amino acid sequence, the *6gt31a* gene encoding Ps6GT31A was found in the *Paenibacillus* sp. 598K genome. The corresponding *Hin*dIII-*Nde*I DNA fragment contained full-length *cit* and *6gt31a* and a partial hypothetical protein-coding gene. The DNA sequence of *6gt31a* was 3846 bp long, and it encoded a 1281-amino-acid protein. BLASTP search of the deduced amino acids indicated that Ps6GT31A has a modular architecture including several functional domains (Fig. 3a). The deduced amino acid sequence of 132 residues was shown to contain a copper amine oxidase-like domain by a BLASTP search.

The two genes, *cit* (106–3024) and *6gt31a* (3084–6929), are closely located in the same orientation in the genome and the partial hypothetical protein-coding gene was observed within positions 7123–7519. The putative promoter regions and terminators were predicted using the Softberry programs BPROM and FindTerm (Solovyev and Salamov 2011). Two possible

promoters and one terminator were predicted. The first putative promoter was located upstream of *cit* at position 86 with a −10 box (AATTCAAAT) at position 71 and a −35 box (ATCAAA) at position 46. The second putative promoter was located between *6gt31a* and the partial hypothetical protein gene at position 7060 with a −10 box (TTTTATATT) at position 7045 and a −35 box (CTGAAT) at position 7028. A putative terminator was identified after the stop codon of *6gt31a* at 239 bp downstream from the stop codon of *6gt31a* and also at 194 bp downstream from the palindromic sequence after *6gt31a*.

The deduced amino acid sequence corresponding to the mature Ps6GT31A (residues 36–1281) resembled 6-glucosyltransferase CtsZ from *Bacillus globisporus* C11 (GenBank accession number BAB88404; 61% identity and 75% similarity) (Aga et al. 2002) (Fig. 3). Residues 36–798 exhibited high similarity to the conserved region of GH31 enzymes: The region showed 28% identity (45% similarity) with *Cellvibrio japonicus* α-xylosidase CjXyl31A (Protein Data Bank [PDB] entry 2XVG) (Larsbrink et al. 2011), 24% identity (42% similarity) with *C. japonicus* oligosaccharide α-1,4-glucosyltransferase CjAgd31B (PDB entry 4B9Y) (Larsbrink et al. 2012), 26% identity (41% similarity) with uncharacterized protein Lmo2446 from *Listeria monocytogenes* (PDB entry 4KMQ), and 23% identity (43% similarity) with α-glucosidase MalA from *Sulfolobus solfataricus* (PDB entry 2G3M) (Ernst et al. 2006). The region including the deduced catalytic domain of Ps6GT31A showed 64% identity (77% similarity) with that of *B. globisporus* CtsZ (Fig. 3b). Residues Asp429 and Asp491 in Ps6GT31A were predicted to be the catalytic nucleophile and the acid/base, respectively. These correspond to Asp433 and Asp495 of *B. globisporus* CtsZ and they are conserved in all of the characterized GH31 enzymes. The NCBI conserved domain search revealed that the enzyme has two family 35 carbohydrate-binding modules (CBM35). In addition, BLASTP search suggested that the remaining C-terminal region is a family 61 carbohydrate-binding module (CBM61). Residues 850–984 and 989–1116, named PsCBM35-1 and PsCBM35-2, respectively, displayed similarities (40% and 37% identity, and 55% and 49% similarity, respectively) to α-(1→6)-glucan-binding module BcCBM35-1 from *P. agaridevorans* T-3040 GH66 CITase (PDB entry 3WNK)(Suzuki et al. 2014). PsCBM35-1 and PsCBM35-2 resembled each other (40% identity, 53% similarity). In addition, residues 1126–1281, named PsCBM61, were 24% identical and 37% similar to β-(1→4)-galactan-binding module TmCBM61 from *Thermotoga maritima* GH53 endo-β-1,4-galactanase (PDB entry 2XOM) (Cid et al. 2010).

Expression and purification of the recombinant protein

The gene encoding mature Ps6GT31A without a secretion signal sequence (residues 1–35) was cloned. Recombinant Ps6GT31A was successfully expressed in *E. coli* and purified as a

single band on SDS-PAGE when visualized by staining with Coomassie Brilliant Blue R-250 (Figure S2, lane 3). The molecular mass of the recombinant protein was estimated to be 136 kDa by SDS-PAGE, which was in good agreement with the native Ps6GT31A and the predicted molecular mass (135.6 kDa) from the amino acid sequence.

Substrate specificity of Ps6GT31A

When Ps6GT31A was incubated with starch, glucose was liberated as the product, suggesting that Ps6GT31A has α-glucosidase activity (Fig. 4a). The effects of pH and temperature on this α-glucosidase activity were investigated using starch as the substrate (Figure S3). The enzyme achieved maximal activity at pH 6.0 and 50°C. The enzyme retained more than 80% of its activity between pH 5.5 and pH 8.0 and at temperatures of less than 50°C. Under the optimal reaction conditions (50°C, pH 6.0), the specific activity of Ps6GT31A toward starch was 1.3 units/mg. The activities of the enzyme toward dextran 40 and pullulan were 0.24 and 0.17 units/mg, respectively. These data suggested that Ps6GT31A exhibits higher hydrolysis activity against α-(1→4)-linked glucan than against α-(1→6)-linked glucan. The enzyme displayed extremely low activity against PNP α-glucopyranoside. Its K_m and k_{cat} values at pH 6.0 at 37°C were 22.2 ± 0.8 mM^{-1} and 0.14 ± 0.01 s^{-1}, respectively. The enzyme did not display any hydrolytic activity against PNP α-mannopyranoside, PNP α-galactopyranoside, and PNP α-xylopyranoside.

The substrate specificity of the enzyme for glucobioses was investigated using G2, kojibiose, nigerose, IG2, trehalose, cellobiose, sophorose, laminaribiose, and gentiobiose as the substrates. The enzyme hydrolyzed α-(1→4)-, α-(1→2)-, α-(1→3)-, and α-(1→6)-linked glucobioses in decreasing order of activity of 100%, 39%, 33%, and 2%, respectively. The enzyme did not hydrolyze trehalose and β-linked glucobioses. In the hydrolysis of panose [α-Glc-(1→6)-α-Glc-(1→4)-Glc], glucose and G2 were generated by Ps6GT31A at the initial stage of the reaction (Fig. 4b). These results revealed that the enzyme liberates glucose from the non-reducing end of the substrate. The k_{cat}/K_m values of Ps6GT31A for the hydrolysis of G2–G7 were 2.3 ± 0.0, 5.6 ± 1.0, 21 ± 3, 40 ± 2, 45 ± 9, and 39 ± 9 $s^{-1}\mu M^{-1}$, respectively. The catalytic efficiency increased as the DP increased from 2 to 5 and remained at the same level at DPs of 5–7, indicating that Ps6GT31A recognizes substrates with lengths comparable to that of G5.

Transglucosylation activity of Ps6GT31A

When Ps6GT31A was incubated with 1% (w/v) G2–G7 or 1% (w/v) IG2–IG7 as the substrates, it produced longer oligosaccharides than each substrate besides degradation products of glucose and short oligosaccharides, indicating that Ps6GT31A also had transglucosylation activity (Figs. 5a and 5b). The enzyme also catalyzed the transglucosylation of kojibiose and nigerose but not trehalose (Fig. 5c). These results indicated that Ps6GT31A had transglucosylation

activity toward α-(1→2)-, α-(1→3)-, α-(1→4)-, and α-(1→6)-linked glucobioses. The transglucosylation/hydrolysis ratios of Ps6GT31A were calculated from the amounts of hydrolytic products, substrate, and transglucosylation products using maltooligosaccharides as the substrates. The transglucosylation/hydrolysis ratios of the enzyme for G2, G3, G4, and G5 were as follows: 5:95, 7:93, 21:79, and 18:82, respectively. The results indicated that maltooligosaccharides with DPs ≥4 were good acceptors of Ps6GT31A relative to short maltooligosaccharides.

The hydrolytic and transglucosylation products of G4 and IG4 produced by the enzyme at the initial stage were analyzed by HPLC-ELSD (Fig. 6). When G4 was used as the substrate, a new larger product with a longer retention time than G4, which was considered a transglucosylation product as denoted by an asterisk in Fig. 6a, was detected in addition to hydrolytic products (glucose and G3). Similarly, the hydrolytic products (glucose and IG3) and the transglucosylation product IG5 were detected when IG4 was used for a substrate (Fig. 6b). Because the transglucosylated product from IG4 was IG5, the new product from G4 should be a transglucosylation product of G4 attached via α-(1→6)-linked glucose. To identify the transglucosylation products of G4, FT-MS and ^{13}C NMR analyses were performed (Figure S4). FT-MS analysis gave rise to one $[M-H]^-$ ion at *m*/*z* 827.3, corresponding to a glucosyl oligosaccharide with a DP of 5 (Figure S4a). The product had ^{13}C NMR signals (D_2O, δ in ppm) of 100.8 [C1 of non-reducing end α-Glc-(1→6)-] and 68.6 [C6 of -α-(1→6)-Glc-α-(1→4)-] [Figure S4b(6)], which are never observed in maltopentaose [Figure S4b(5)]. These results revealed that the enzyme transferred one glucose to the non-reducing end of substrate G4 and elongated oligosaccharides with α-(1→6)-linkages.

Transglucosylation products by Ps6GT31A and CI production by CITase

Ten fractions of hydrolysis and transglucosylation reaction products from G5 after 24-h Ps6GT31A treatment were obtained by HPLC separation and named fractions 1–10 (Fig. 7a). Fractions 1–3 were identified as glucose, G2, and G3, respectively, on the basis of the retention times. FT-MS analysis of fractions 2–10 revealed $[M+H]^+$ ions at *m*/*z* 343.1, 505.1, 667.2, 829.3, 991.2, 1153.4, 1315.5, 1477.7, and 1639.8, corresponding to glucooligosaccharides with DP2–10 (Table 1), respectively. To examine whether CITase produces CI from these products, fractions 4–10 were individually incubated with CITase. Then the reaction mixture was incubated with glucoamylase and highly branched dextran hydrolase. The remaining linear oligosaccharides are digested completely to glucose with this treatment, and intact cyclic oligosaccharides are left. When fractions 4 and 5 were used as the substrates, CI production was not detected. By contrast, CI production was detected when CITase was incubated with fractions 6–10 (Table 1). Figure 7b shows CI production by CITase when the enzyme was incubated with fraction 7 as the substrate.

CITase mainly produced CI-7–9 from fraction 7. When fractions 6–10 were used as the substrates, the amounts of CI produced by CITase, which were the sums of CI-7–9, were 53.7 ± 0.1, 69.0 ± 4.4, 139.6 ± 2.7, 122.5 ± 4.3, and 197.8 ± 6.0 μM (Table 1), respectively. CITase produced larger amounts of CI when larger molecules of fractions 8–10 were used as the substrates. The results indicated that CITase can utilize the Ps6GT31A-transglucosylation products from maltooligosaccharides as substrates for producing CIs.

Fractions 4–10 were individually treated with oligo-1,6-glucosidase and/or *B. stearothermophilus* α-glucosidase and subsequently analyzed by HPLC-ELSD. Oligo-1,6-glucosidase specifically hydrolyzes the non-reducing end of α-(1→6)-glucosidic linkages of isomaltooligosaccharides. By contrast, *B. stearothermophilus* α-glucosidase specifically hydrolyzes α-(1→4)-glucosidic linkages from the non-reducing end of oligosaccharides. When fraction 7 was treated with oligo-1,6-glucosidase, glucose, G4, G5, and two additional peaks were detected by HPLC (Fig. 7c). This indicated that fraction 7 consisted of a mixture of two major heptasaccharides, α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{IV}G4 and α-Glc-(1→6)-α-Glc-(1→6)-^{V}G5 (Table 1, Roman numerals indicate the substituted residues in ascending order starting from the reducing end). When fraction 7 was treated with a mixture of oligo-1,6-glucosidase and *B. stearothermophilus* α-glucosidase, it was almost completely hydrolyzed to glucose (Fig. 7c, bottom panel). Similarly, fractions 4–10 were also completely hydrolyzed to glucose by these enzymes (data not shown). These results indicated that fractions 4–10 contained only α-(1→6)- and/or α-(1→4)-linked glucoses. The structures and ratios of the major reaction products estimated by enzymatic treatment and the amount of CI produced by CITase when they were used as substrates are shown in Table 1. The results of oligo-1,6-glucosidase treatment indicated that Ps6GT31A created α-(1→6)-linked glucosyl moieties at the non-reducing end of maltooligosaccharide through successive transglucosylation reactions. Actually, all transglucosylation products isolated in fractions 4–10 are such types of glucooligosacharide. CI production by CITase was observed in the case of fractions 6–10 but not fraction 4 or 5. The amounts of CIs produced from fractions 8–10 were greater than those produced from fractions 6 and 7.

Discussion

Glycoside hydrolases and carbohydrate-binding modules are classified into families in the Carbohydrate-Active EnZymes (CAZy) database according to the similarity between their amino acid sequences (Lombard et al. 2013). GH31 includes α-glucosidase (EC 3.2.1.20), α-1,3-glucosidase (EC 3.2.1.84), sucrase-isomaltase (EC 3.2.1.48, EC 3.2.1.10), and α-xylosidase (EC 3.2.1.177). It also includes some transferases involved in the rearrangement of α-glucans, such as cycloalternan synthetic enzymes CtsY and CtsZ from *Bacillus* sp. (Kim et al. 2003; Nishimoto et al. 2002) and the glycogen synthetic enzyme oligosaccharide α-1,4-glucosyltransferase from *C. japonicus* (Larsbrink et al. 2012). GH31 enzymes act with a retaining mechanism; therefore, GH31 includes α-glucosidases with strong transglucosylation activity (Kato et al. 2002; Ota et al. 2009).

Ps6GT31A was isolated from *Paenibacillus* sp. 598K culture supernatant as the only enzyme that produces a substrate for CITase to synthesize CIs using starch or maltooligosaccaharides. It was revealed to be a GH31 enzyme with broad-specificity α-glucosidase activity and strong α-(1→6)-transglucosylation activity. The enzyme transferred one glucosyl residue from the non-reducing end of maltooligosaccharide to the non-reducing end of another molecule to produce an α-(1→6)-glucosyl linkage. The α-(1→6)-glucosyl moiety was then elongated by the successive addition of glucose to the non-reducing end of the growing chain via the transglucosylation activity of the enzyme in these fractions. The reaction products from G5 generated by Ps6GT31A summarized in Table 1 indicate that fraction 4 includes α-Glc-(1→6)-^{III}G3 and that fraction 5 includes α-Glc-(1→6)-^{IV}G4 and α-Glc-(1→6)-α-Glc-(1→6)-^{III}G3. These results suggest that the α-(1→6)-glucosyl moiety with DP 2–3 was introduced at the non-reducing end of the oligosaccharide via successive transglucosylation of the enzyme. The results for oligo-1,6-glucosidase-treated fractions 6 and 7 indicate that the enzyme produced hexasaccharides containing α-(1→6)-glucosyl moieties with DP 2–4 and heptasaccharides containing α-(1→6)-glucosyl moieties with DP 3–4. In the case of fractions 8–10, oligosaccharides containing α-(1→6)-glucosyl moieties with DP of ≥5 were found. When CITase was incubated with fractions 4–10 as the substrates, CI production was observed for fractions 6–10 but not for fraction 4 or 5. These results indicate that the IG4 component at the non-reducing end of the substrate was necessary for CI production, which is supported by the previous finding that IG4 is the smallest substrate of CITase for CI production (Suzuki et al. 2012). Because fractions 8–10 contained oligosaccharides with longer α-(1→6)-glucosyl chains than IG4, fractions 8–10 are likely to be better substrates for CITase than fractions 6 and 7, resulting in higher levels of CI production.

The deduced amino acid sequence of mature Ps6GT31A resembled 6-

glucosyltransferase from *B. globisporus* C11, which is involved in cycloalternan synthesis (Nishimoto et al. 2002). The enzyme catalyzes the α-(1→6)-transglucosylation of one glucosyl residue to the non-reducing end of maltooligosaccharide to produce α-Glc-(1→6)-maltooligosaccharide, but not the successive α-(1→6)-transglucosylation to produce the α-(1→6)-glucosyl chain. From the perspective of the enzymatic action of α-(1→6)-transglucosylation, this enzyme is similar to Ps6GT31A, but Ps6GT31A was distinctly different from *B. globisporus* 6-glucosyltransferase in terms of the number of transglucosylated glucose residues.

A BLAST search revealed that Ps6GT31A has at least three CBMs, PsCBM35-1, CBM35-2, and PsCBM61, on its C-terminal side (Fig. 3a). The deduced amino acid sequences of PsCBM35-1 and PsCBM35-2 resembled the α-(1→6)-glucan-binding module BcCBM35-1 of *P. agaridevorans* T-3040 CITase (Fig. 3c). Aromatic residues such as tryptophan and histidine, which bind to glucose at the BcCBM35-1 sugar-binding site, were conserved in PsCBM35-1 and PsCBM35-2 (His875, Trp943, His1009, and Trp1077) of Ps6GT31A (Suzuki et al. 2014). These aromatic residues were also conserved in CBMs of 6-glucosyltransferase (Fig. 3c). There are four different kinds of CBM35, which show affinity toward α-glucan, mannan, xylan, or β-1,3-galactan (Bolam et al. 2004; Ichinose et al. 2005; Suzuki et al. 2015). PsCBM35-1 and PsCBM35-2 would be expected to be members of the α-glucan-binding subfamily. The C-terminal PsCBM61 displayed similarity with β-(1→4)-galactan-binding module TmCBM61 from *T. maritima* endo-β-1,4-galactanase (Cid et al. 2010) (Fig. 3d). TmCBM61 has three tryptophans in the β-(1→4)-galactan-binding site. Typ508 in TmCBM61 was replaced by histidine in Ps6GT31A (His1181) and phenylalanine in 6-glucosyltransferase (Phe1185). The other residues were not conserved in Ps6GT31A and 6-glucosyltransferase. Although further analysis of Ps6GT31A will be necessary to understand α-glucan recognition, there are some distinct differences in sugar-binding sites between α-glucan-binding and β-1,3-glucan-binding CBM61s.

In this paper, we describe that *Paenibacillus* sp. 598K produced CI without dextran via the contribution of Ps6GT31A. The *6gt31a* gene is located immediately downstream from the *cit* gene in the *Paenibacillus* sp. 598K genome and there are no promoter or palindromic sequences between these genes. The *6gt31a* gene and the downstream gene are at the distance of 241 bp from each other and are dissociable by a palindromic sequence. Therefore, only *cit* and *6gt31a* should be regulated by the same promoter and co-expressed. The CITase activity of the bacterium was induced by pullulan, B1299 glucan, B1355 glucan, and dextran, but not by other sugars including isomaltooligosaccharides. The α-glucan containing an α-(1→6)-linkage should be necessary, but the short DP of α-(1→6)-linked glucoses does not seem to be sufficient for CI

production in *Paenibacillus* sp. 598K. Glucose addition in dextran 40-supplemented culture broth had no effect on CITase activity. In the case of *P. agaridevorans* T-3040, activity of the 135-kDa protein (most probably 6GT31A) was induced by starch and dextran, and suppressed by the addition of glucose to the culture broth (Funane et al. 2014). Conversely, its CITase was reported to be induced by starch, dextran, and even by small molecules of isomaltooligosaccharides of DP = 2–3, and the addition of glucose did not affect CITase production upon growth with dextran or isomaltooligosaccharides, but it was almost completely inhibited upon growth with starch. The *cit* and *6gt31a* genes are likely to be expressed simultaneously and regulated in the same way in *Paenibacillus* sp. 598K, whereas these genes are considered to be differently expressed and regulated in *P. agaridevorans* T-3040. Despite these differences, the growth patterns of *P. agaridevorans* T-3040 and *Paenibacillus* sp. 598K showed some similarity. These bacterial strains grow well with dextran as the sole carbon source but less well with starch, showing a long lag phase of around 1 day (Figure S1) (Funane et al. 2014). It seems that dextran is a good carbon source for these CI-producing bacteria for both their growth and CI production, but dextran is usually produced by other bacteria such as dextransucrase-producing lactic acid bacteria or dextran dextrinase-producing acetic acid bacteria, as mentioned previously. While CI-producing bacteria use dextran as a carbon source, they induce another CI-producing enzymatic system, which enables them to produce CIs from starch. One possible function of CIs for these bacteria is as their exclusive carbon source for nutritional purposes. They may have multiple ways of producing CIs from different materials for their survival, but further investigations will be required to understand the meaning of CI production for the bacterial strains.

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Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

This article does not describe any studies on human participants or animals performed by any of the authors.

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Figure legends

Fig. 1 CI-producing activity against dextran and starch in the culture supernatants of a medium containing various carbon sources

White bar, CITase activity with 2% (w/v) dextran 40; black bar, CITase activity with 2% (w/v) starch. The error bars indicate the standard deviation of triplicate experiments. None, no carbon source; Mat M-22 starch, Matsunorin M-22 starch; B1299 glucan, *L. mesenteroides* NRRL B-1299 glucan; B1355 glucan, *L. mesenteroides* NRRL B-1355 glucan; Dextran 40+Glc, 2% (w/v) dextran 40 with 1% (w/v) glucose.

Fig. 2 Purification of Ps6GT31A by Resource Q chromatography

CITase activities against dextran 40 and starch in each fraction were measured as described previously (Suzuki et al. 2012). White circle, CITase activity when dextran 40 was used as the substrate; solid square, CI-producing activity when starch was used as the substrate; solid triangle, CI-producing activity when starch was used as the substrate, supplemented with fraction no. 49 (native CITase) added to the fractions at a 1:1 ratio (v/v).

Fig.3 Primary structure of Ps6GT31A

(a) Schematic drawing of the Ps6GT31A molecular architecture. GH31 conserved region, highly conserved region in glycoside hydrolase family 31; PsCBM35-1 and PsCBM35-2, carbohydrate-binding module family 35; PsCBM61, carbohydrate-binding module family 61. The numbers above the bars denote the amino acid residue numbers. (b) Partial sequence comparison with Ps6GT31A and *B. globisporus*C11 6-glucosyltransferase (Bg6GT) (Aga et al. 2002). The alignment was performed using ClustalW2 (Larkin et al. 2007). Identical amino acid residues are in black boxes and similar residues in gray boxes. The asterisks indicate the putative catalytic residues. (c) Sequence alignment of CBM35 among PsCBM35-1, PsCBM35-2, *B. globisporus* C11 6-glucosyltransferase BgCBM35-1, BgCBM35-2, and *Paenibacillus agaridevorans* CITase BcCBM35-1 (Uniprot ID P94286) (Suzuki et al. 2014). The residues corresponding to BcCBM35-1 sugar-binding sites are boxed. (d) Sequence alignment of CBM61s among PsCBM61, 6-glucosyltransferase BgCBM61, and *T. maritima* endo-β-1,4-galactanase TmCBM61 (Uniprot ID Q9X0S8) (Cid et al. 2010). Open circles (○) indicate residues consisting of TmCBM61 sugar-binding sites.

Fig. 4 HPLC-ELSD analysis of the hydrolyzed products of (a) starch and (b) panose generated by Ps6GT31A

Ps6GT31A (120 nM) was incubated with 0.5% (w/v) starch in 30 mM Tris-malate buffer (pH 6.0) at 50°C. For panose, the enzyme was incubated with 0.1 mM substrate in 40 mM Tris-malate buffer (pH 6.0) at 50°C. Reaction products were analyzed by HPLC-ELSD. An acetonitrile–water (60:40, v/v) mixture was used as a mobile phase. Abbreviations: Glc, glucose; G2, maltose; Pa, panose.

Fig. 5 TLC analysis of the reaction products of glucooligosaccharides generated by Ps6GT31A

(a) S1, glucose and G2–G7 (standards); 1, glucose used as the substrate; 2, G2 used as the substrate; 3, G3 used as the substrate; 4, G4 used as the substrate: 5, G5 used as the substrate; 6, G6 used as the substrate; 7, G7 used as the substrate. (b) S2, IG2–IG7 (standards); 1, glucose used as the substrate; 2, IG2 used as the substrate; 3, IG3 used as the substrate; 4, IG4 used as the substrate; 5, IG5 used as the substrate; 6, IG6 used as the substrate; 7, IG7 used as the substrate; E, enzyme only. (c) S3, glucose (standard); T, trehalose used as the substrate; K, kojibiose used as the substrate; N, nigerose used as the substrate; –, no enzyme; +, incubated with the enzyme. Ps6GT31A (350 nM) was incubated with 1% (w/v) substrate in 40 mM Tris-malate buffer (pH 6.0) at 50°C for 24 h. The products were examined by TLC using silica gel 60 F_{254} in a solvent system of 1-butanol:acetic acid:water at a ratio of 2:1:1 (a and b) or acetonitrile:water at a ratio of 4:1 (c).

Abbreviations: Glc, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose; IG2, isomaltose; IG3, isomaltotriose; IG4, isomaltotetraose; IG5, isomaltopentaose; IG6, isomaltohexaose; IG7, isomaltoheptaose.

Fig. 6 HPLC-ELSD analysis of the reaction products of (a) maltotetraose and (b) isomaltotetraose produced by Ps6GT31A

Reaction products of maltotetraose (a) and isomaltotetraose (b) at the initial stage were analyzed. The new product generated by the enzyme is indicated by an asterisk.

Abbreviations: Glc, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; IG2, isomaltose; IG3, isomaltotriose; IG4, isomaltotetraose; IG5, isomaltopentaose. An acetonitrile–water (60:40, v/v) mixture was used as the mobile phase.

Fig. 7 HPLC-ELSD analysis of the reaction products of maltopentaose produced by Ps6GT31A and the hydrolytic products produced by glucosidases

(a) Reaction products of maltopentaose produced by Ps6GT31A. Ten fractions were named fractions1–10. (b) CI production by CITase using fraction 7 as the substrate. Fraction 7 was

incubated with (+) or without (−) CITase. ~~Reaction~~ The reaction products were digested with glucoamylase and highly branched dextran hydrolase, and they were analyzed by HPLC-ELSD ~~after glucoamylase and highly branched dextran hydrolase enzymatic digestion of the remaining linear glucooligosaccharides,~~ as described previously (Funane et al. 2014). (c) Enzymatic hydrolysis of fraction 7. Top panel, Standards (glucose and maltooligosaccharides); second panel, fraction 7 (untreated); third panel, oligo-1,6-glucosidase-treated (/α-1,6); bottom panel, oligo-1,6-glucosidase and *B. stearothermophilus* α-glucosidase-treated (/α-1,6/α-1,4). Abbreviations: Glc, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose.

Table 1

Reaction products of G5 produced by Ps6GT31A as indicated in Figure 7a and CI production from the enzyme product by CITase

Fraction number	Observed *m/z* $[M+H]^+$	DP	[a]Major products (%)	[b]CI produced (μM)
2	343.1	2	G2 (100)	Not determined
3	505.1	3	G3 (100)	Not determined
4	667.2	4	α-Glc-(1→6)-^{III}G3 (19) G4 (13)	Not detected
5	829.3	5	α-Glc-(1→6)-α-Glc-(1→6)-^{III}G3 (16) α-Glc-(1→6)-^{IV}G4 (23) G5 (4)	Not detected
6	991.2	6	α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{III}G3 (4) α-Glc-(1→6)-α-Glc-(1→6)-^{IV}G4 (55) α-Glc-(1→6)-^{V}G5 (13)	53.7 ± 0.1
7	1153.4	7	α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{IV}G4 (17) α-Glc-(1→6)-α-Glc-(1→6)-^{V}G5 (49)	69.0 ± 4.4
8	1315.5	8	α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{IV}G4 (14) α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{V}G5 (23)	139.6 ± 2.7
9	1477.7	9	α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{IV}G4 (11) α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{V}G5 (25)	122.5 ± 4.3
10	1639.8	10	α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α- (9)	197.8 ± 6.0

			Glc-(1→6)-^{IV}G4 (20) α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)- ^{V}G5	

[a]The value is the average of two independent assays. [b]Results are presented as the mean ± S.E. The assay was performed in triplicate.

Roman numerals indicate the substituted residues in ascending order stating from the reducing end.

Paenibacillus sp. 598K 6-α-glucosyltransferase is essential for cycloisomaltooligosaccharide synthesis from α-(1→4)-glucan

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Abstract

Paenibacillus sp. 598K produces cycloisomaltooligosaccharides (cyclodextrans) from starch, even in the absence of dextran. Cycloisomaltooligosaccharide glucanotransferase synthesizes cycloisomaltooligosaccharides exclusively from an α-(1→6)-consecutive glucose chain consisting of at least four molecules. Starch is not a substrate of this enzyme. Therefore, we predicted that the bacterium possesses another enzyme system for extending α-(1→6)-linked glucoses from starch, which can be used as the substrate for cycloisomaltooligosaccharide glucanotransferase, and identified the transglucosylation enzyme Ps6GT31A. We purified Ps6GT31A from the bacterial culture supernatant, cloned its corresponding gene, and characterized the recombinant enzyme. Ps6GT31A belongs to glycoside hydrolase family 31, and it liberates glucose from the non-reducing end of the substrate in the following order of activity: α-(1→4)- > α-(1→2)- > α-(1→3)- > α-(1→6)-glucobiose and maltopentaose > maltotetraose > maltotriose > maltose. Ps6GT31A catalyzes both hydrolysis and transglucosylation. The resulting transglucosylation compounds were analyzed by high-performance liquid chromatography and mass spectrometry. Analysis of the initial products by ^{13}C nuclear magnetic resonance spectroscopy revealed that Ps6GT31A had a strong α-(1→4) to α-(1→6) transglucosylation activity. Ps6GT31A elongated α-(1→6)-linked glucooligosaccharide to at least a degree of polymerization of 10 through a successive transglucosylation reaction. Eventually, cycloisomaltooligosaccharide glucanotransferase creates cycloisomaltooligosaccharides using the transglucosylation products generated by Ps6GT31A as the substrates. Our data suggest that Ps6GT31A is the key enzyme to synthesize α-(1→6)-glucan for cycloisomaltooligosaccharide production in dextran-free environments.

Keywords: cycloisomaltooligosaccharide, 6-α-glucosyltransferase, glycoside hydrolase family 31, *Paenibacillus* sp. 598K, starch

Introduction

Cycloisomaltooligosaccharide (cyclodextran, CI) is a bacterial cyclic oligosaccharide consisting of α-(1→6)-linked glucosyl residues (all of the sugars in the present study are in the D-configuration unless otherwise specified) (Funane et al. 2008; Oguma et al. 1993). CI is highly hydrophilic, and it is an anti-plaque carbohydrate with strong inhibitory activity against streptococcal glucansucrases (Kobayashi et al. 1995). CI forms an inclusion complex with insoluble and/or unstable compounds; that is, CI-7 and CI-8 (CI-*n*, where *n* is the number of glucose molecules) solubilize C_{60} and C_{70} fullerenes (Jina et al. 1996), and CI-10 stabilizes Victoria blue B (Funane et al. 2007).

To date, three CI-producing bacteria, *Paenibacillus agaridevorans* T-3040 {formerly *Bacillus circulans* T-3040 [FERMBP-4132 (NBRC)]; 16S rRNA accession number, LC042199, K. Ochi, personal communication}, *Paenibacillus* sp. 598K, and *B. circulans* U-155, have been reported (Funane et al. 2008; Oguma et al. 1993, 2014). When the bacteria are grown in the presence of dextran, expression of the extracellular enzyme cycloisomaltooligosaccharide glucanotransferase (CITase; EC 2.4.1.248) is induced and CIs are simultaneously produced from dextran by CITase in the culture supernatants (Oguma et al. 1994, 2014; Suzuki et al. 2012). CITase catalyzes the intramolecular (cyclization) and intermolecular (disproportionation, coupling) transglycosylation and hydrolysis reactions of α-(1→6)-glucan (dextran) (Oguma et al. 1994) and isomaltooligosaccharides [degree of polymerization (DP) ≥4] (Suzuki et al. 2012).

Dextran is one of the exopolysaccharides produced by lactic acid bacteria such as *Leuconostoc*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, and *Weissella* (Monsan et al. 2001; Torino et al. 2015), as well as *Rhizopus* spp. (Samkpal et al. 2001). They produce extracellular dextransucrase (EC 2.4.1.5) that synthesizes dextran from sucrose (Leemhuis et al. 2013). Another dextran-producing bacterium, *Gluconobacter oxydans*, produces dextran dextrinases (EC 2.4.1.2) that synthesize dextran from maltodextrins (Naessems et al. 2005). As no reports of dextran synthase in CI-producing bacteria have been published, there were two possibilities: CI-producing bacteria synthesize dextran using an unknown enzyme system by themselves, or CI-producing bacteria utilize dextran produced by other dextran-producing bacteria. Recently, we reported that *P. agaridevorans* T-3040 produces CI from starch even in the absence of dextran (Funane et al. 2014). The bacterium autonomously produces CI from starch, and a 135-kDa protein possessing transglucosylation activity with maltooligosaccharides was assumed to be required for CI production from starch, although the details remain unclear.

In this study, *Paenibacillus* sp. 598K was cultured in a medium containing various carbon sources, and CI-producing activity was investigated to determine whether the bacterium possesses

an enzyme system for CI production without dextran. We found a key enzyme for α-(1→6)-glucan synthesis from starch and named it Ps6GT31A. It belongs to glycoside hydrolase family 31 (GH31), which consists of diverse enzymes such as α-glucosidase (EC 3.2.1.20), α-xylosidase (EC 3.2.1.177), and oligosaccharide α-1,4-glucosyltransferase (EC 2.4.1.161). In this study, we report the detailed characteristics of Ps6GT31A and discuss its involvement in CI production from starch.

Materials and Methods

Substrates

Pregelatinized starch (Matsunorin M-22 starch) was purchased from Matsutani Chemical Industry Co., Ltd. (Itami, Japan). Dextrin was obtained from Becton, Dickinson and Company (Sparks, USA), and dextran 40 was acquired from GE Healthcare, UK, Ltd. (Little Chalfont, UK). Maltose (G2), maltotriose (G3), pullulan, which commonly consists of α-(1→6) linked maltotriose, kojibiose, nigerose, and cellobiose were procured from Wako Pure Chemical Industries (Osaka, Japan). Maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), maltoheptaose (G7), and Fujioligo G67 (maltohexaose- and maltoheptaose-rich maltooligosaccharides) were purchased from Nihon Shokuhin Kako, Co., Ltd. (Tokyo, Japan). Isomaltooligosaccharides (IG2–IG7; of which the number represents glucose molecules) were acquired from Seikagaku Co. (Tokyo, Japan). Isomalto 500 (a mixture of IG2, IG3, and panose) was obtained from Showa Sangyo Co., Ltd. (Tokyo, Japan). Trehalose was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and gentiobiose, sophorose, panose, *p*-nitrophenyl (PNP) α-glucopyranoside, PNP α-mannopyranoside PNP α-galactopyranoside, and PNP α-xylopyranoside were procured from Sigma Chemical Company (St. Louis, MO, USA). Laminaribiose was obtained from Megazyme International (Bray, Ireland). Standard CIs (CI-7–CI-9) were acquired from C-I Bio Ltd. (Tomigusuku, Japan). Glucans from *Leuconostoc mesenteroides* NRRL B-1299 [B1299 glucan, which contains α-(1→6) and α-(1→2) linkages] (Brison et al. 2012) and NRRL B-1355 [B1355 glucan, which contains α-(1→6) and α-(1→3) linkages] (Côte and Robyt 1982) were kindly donated by Dr. M. Kobayashi.

CI-producing activity against dextran and starch in the culture supernatants of a medium containing various carbon sources

Paenibacillus sp. 598K [FERM P-19604(NBRC)] was cultured in 2 mL of Luria–Bertani (LB) broth, which was supplemented with or without 2% (w/v) carbohydrate sources, namely, glucose, sucrose, Fujioligo G67, dextrin, Matsunorin M-22 starch, Isomalto 500, nigerose, kojibiose, pullulan, B1299 glucan, B1355 glucan, dextran 40, or 2% (w/v) dextran 40 with 1% (w/v) glucose at 30°C for 3 days with shaking at 200 min^{-1}. Each of the culture broths was centrifuged at 10,000 × *g* for 10 min, and the supernatants were collected. Each culture supernatant was desalted and concentrated using an Amicon Ultra filter (10,000 MWCO; Merck Millipore, Billerica, MA, USA), and CI-producing activity against dextran and starch was determined as described previously (Funane et al. 2014). Briefly, the sums of the amounts of CI-7, CI-8, and CI-9 were quantified by high-performance liquid chromatography with an evaporative light scattering detection system (HPLC-ELSD; LC Workstation Class-VP;

Shimadzu, Co., Kyoto, Japan) using a TSK gel Amide-80 column (4.6 × 250 mm; Tosoh, Co., Tokyo, Japan). The experiments were performed in triplicate. One unit of CITase activity was defined as the amount of enzyme that released 1 μmol of the sum of CI-7, CI-8, and CI-9 per minute.

Purification and sequence analysis of native Ps6GT31A

Paenibacillus sp. 598K was grown in 400 mL of LB medium containing 2% (w/v) dextran 40 in a baffled flask at 30°C for 3 days with shaking at 160 min^{-1}. The culture was centrifuged at 10,000 × *g* for 10 min. Ps6GT31A was purified from the supernatant by precipitation with 20%–60% saturated ammonium sulfate followed by Resource Q chromatography (GE Healthcare) twice. The proteins were dialyzed against 20 mM Tris-HCl (pH 8.0). The bound proteins were eluted using a 0–600 mM NaCl linear gradient. The CITase activity against dextran 40 and starch in each fraction was measured as described above. Purified protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a PVDF membrane. The sequence of the 23 N-terminal amino acids was determined using an HP G1005A protein sequencer (Hewlett-Packard, Palo Alto, CA, USA).

Because the DNA fragment encoding the N-terminal amino acid sequence was found in the 5269-bp *Hin*dIII DNA fragment (GenBank accession no. DJ083453), which contained the full-length *cit* gene (GenBank accession no. AB685169) reported previously (Suzuki et al. 2012), the *Hin*dIII DNA fragment was used as a probe for selecting the Ps6GT31A-encoding gene *6gt31a* from genomic DNA libraries. The resultant 7519-bp *Hin*dIII–*Nde*I DNA fragment containing full-length *cit* and *6gt31a* and partial hypothetical protein-coding genes were sequenced.

Expression and purification of recombinant Ps6GT31A

The gene encoding mature Ps6GT31A (Ala36–Pro1281) without a secretion signal sequence (Met1–Ala35) was amplified from the genomic DNA by PCR using the following primers: forward, 5′-CATATGGCCGGGCTCGGCAATG-3′; and reverse, 5′-GGATCCTTAAGGCGCTCGGGTGAG-3′. The amplified DNA was cloned into pET15b vectors (Novagen, Inc., Madison, WI, USA) at *Nde*I and *Hin*dIII restriction enzyme sites (underlined). *Escherichia coli* BL21 (DE3) cells (Novagen) harboring the expression plasmid were cultured, and the expression of N-terminal fusion His-tagged Ps6GT31A was induced with 0.1 mM isopropylβ-thiogalactopyranoside for 24 h at 16°C. The cells were harvested and resuspended in 50 mM potassium phosphate-NaOH buffer (pH 7.2), followed by sonication for 5 min. After centrifugation to remove insoluble material, the supernatant was loaded onto a 1-mL HisTrap HP column (GE Healthcare) equilibrated with the same buffer. The bound enzyme was eluted with 50 mM potassium phosphate-NaOH buffer (pH 7.2) containing 250 mM imidazole. The eluted

enzyme, identified by SDS-PAGE as a 136-kDa protein, was dialyzed against 20 mM Tris-HCl (pH 8.0). It was further purified by being loaded onto a Mono Q column (GE Healthcare) equilibrated with the same buffer and eluted with a linear gradient of 0–1 M NaCl in 20 mM Tris-HCl (pH 8.0). Two major peaks of Ps6GT31A were obtained. The peak eluted at the lower salt concentration was the monomer and the one eluted at the higher salt concentration was the dimer determined by gel filtration chromatography using a Superose 12 column (GE Healthcare). The former fraction was dialyzed against 20 mM Tris-HCl (pH 8.0) and used as the purified enzyme. The protein concentration was determined by measuring absorbance at 280 nm, assuming that an absorbance value of 1.0 indicated a concentration of 0.48 mg/mL (molar extinction coefficient = 290,270 $M^{-1} \cdot cm^{-1}$).

Detection of mono- and oligosaccharides

Hydrolytic and transglucosylation products generated by Ps6GT31A were detected by thin-layer chromatography (TLC; TLC Silica gel 60 F_{254} plates; Merck Millipore) with an appropriate solvent or by HPLC-ELSD with a TSK gel Amide-80 column (4.6 × 250 mm) as described above.

α-Glucosidase activity of Ps6GT31A

The reaction mixture consisted of 300 μL of 0.1 M Tris-malate buffer (pH 6.0), 500 μL of 1% (w/v) starch, and 100 μL of 0.002% (w/v) L-rhamnose (internal standard). After pre-incubation at 50°C for 10 min, 100 μL of the enzyme preparation was added, and the reactions were performed at 50°C. At regular time intervals, 100-μL aliquots of the reaction mixture were obtained. After heat incubation at 100°C for 5 min, α-glucosidase activity was determined by the released glucose quantified by HPLC-ELSD using the same column as above with an acetonitrile–water (60:40, v/v) mixture as the mobile phase at 30°C and a flow rate of 1 mL/min. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol glucose per minute from the substrate under these conditions. The substrate specificity of Ps6GT31A for polysaccharides was also determined by the amounts of glucose released.

The effect of temperature on the enzyme activity was examined at a set temperature instead of 50°C for 10 min. Concerning the effect of temperature on enzyme stability, the enzyme (12 nM) dissolved in 20 mM Tris-HCl buffer (pH 8.0) was incubated at a set temperature for 1 h. Then, it was used for the enzyme assay at 50°C for 10 min. The effects of pH on enzyme activity were examined using 50 mM sodium acetate buffer (pH 4.0–5.5) and 0.1 M Tris-malate buffer (pH 5.5–8.0), as well as Atkins–Pantin buffer (0.2 M boric acid/0.2 M KCl/0.2 M Na_2CO_3, pH 8.0–11.0) substituted for 0.1 M Tris-malate buffer (pH 6.0). Regarding the effects of pH stability on enzyme activity, the enzyme was incubated at 37°C for 1 h, and then it was used for the enzyme assay as described above.

The substrate specificity of the enzyme for glucobioses was analyzed using G2 [α-Glc-(1→4)-Glc], kojibiose [α-Glc-(1→2)-Glc], nigerose [α-Glc-(1→3)-Glc], IG2 [α-Glc-(1→6)-Glc], trehalose [α-Glc-(1↔1)-α-Glc], cellobiose [β-Glc-(1→4)-Glc], sophorose [β-Glc-(1→2)-Glc], laminaribiose [β-Glc-(1→3)-Glc], and gentiobiose [β-Glc-(1→6)-Glc]. Briefly, an aliquot of enzyme (0.31–2.0 μM) was incubated with 100 μM substrate in 30 mM Tris-malate buffer (pH 6.0) for up to 120 min at 50°C. The amount of each product was quantified by HPLC-ELSD. To assess the catalytic efficiency of the enzyme for maltooligosaccharides, the enzyme (4–312 nM) was incubated with 100 μM substrate. Progress curves of oligosaccharide cleavage were used to determine k_{cat}/K_m. The activity for PNP glycosides was determined as follows. The reactions were performed in 30 mM Tris-malate buffer (pH 6.0) containing 1–5 mM substrates and 0.7 μM enzyme at 37°C. The amount of *p*-nitrophenol released was determined from the absorbance at 400 nm (molar extinction coefficient = 2,213 $M^{-1}\cdot cm^{-1}$). The assay was performed in triplicate. The kinetic parameters k_{cat} and K_m were determined using Eadie–Hofstee plots.

Transglucosylation activity of Ps6GT31A

The transglucosylation/hydrolysis ratios for maltooligosaccharides were determined as follows. The reactions were performed in 30 mM Tris-malate buffer (pH 6.0) containing 0.4 mM substrates and 15 nM–1.4 μM enzyme at 50°C for up to 20 min. The amounts of substrate (G_n, where n is the number of glucose molecules), hydrolysis products (glucose and G_{n-1}), and transglucosylation products (G_{n+1}) were quantified by HPLC-ELSD. To analyze the reaction products of G4 and IG4 at the initial stage, the enzyme (60 nM for G4, 0.8 μM for IG4) was incubated with the substrate (0.8 mM G4 or 1.7 mM IG4) in 30 mM Tris-malate buffer (pH 6.0) at 50°C.

Reaction products of G4 at the initial stage were purified by HPLC with a refractive index detector (RID-10A; Shimadzu) and a TSKgel Amide-80 column (21.5 × 300 mm; Tosoh) using an acetonitrile–water (55:45, v/v) mixture as the mobile phase at 30°C and a solvent flow rate of 5 mL/min. The lyophilized products were analyzed by Fourier transform mass spectrometry (FT-MS) and ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy. The mass spectra were determined using a JMS HX-100/100A mass spectrometer (JEOL, Ltd., Tokyo, Japan) via fast-atom bombardment ionization. The ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin, Karlsruhe, Germany) at 298K.

Enzymatic treatment of reaction products generated by Ps6GT31A

Reaction products were generated by Ps6GT31A (35 nM) reacting with 1% (w/v) G5 and 0.1% (w/v) bovine serum albumin in water at 37°C for 24 h and separated into 10 fractions by HPLC (fractions 1–10). Nine fractions (fractions 2–10) were analyzed by FT-MS. Each product

(0.1 mg) was incubated with oligo-1,6-glucosidase (Megazyme, 10 units) in 20 mM sodium acetate buffer (pH 4.5) or a mixture of oligo-1,6-glucosidase (5 units) and α-glucosidase from *Bacillus stearothermophilus* (Megazyme, 5 units) in 20 mM Tris-malate buffer (pH 6.0) at 40°C for 20 h. The hydrolytic products were analyzed by HPLC-ELSD. The assay was performed in duplicate.

CI production by CITase from transglucosylation products generated by Ps6GT31A

Fractions 2–10 (5 mM) mentioned above were individually incubated with 100 nM recombinant *Paenibacillus* sp. 598KCITase (Suzuki et al. 2012) in 30 mM Tris-malate buffer (pH 6.0) at 37°C for 24 h and boiled at 100°C for 10 min. CI-producing activity was measured as the total production of CI-7, CI-8, and CI-9 after enzymatic digestion of the remaining linear glucooligosaccharides with highly branched dextran hydrolase and glucoamylase, as described previously (Funane et al. 2014; Suzuki et al. 2012).

GenBank accession numbers

The partial sequence of the 16S rRNA gene of *Paenibacillus* sp. 598K has been deposited with GenBank under the accession number LC155798. The *Hin*dIII-*Nde*I nucleotide sequence of the 7519-bp segment containing full-length *cit* and *6gt31a* genes and partial hypothetical protein-coding gene was deposited in GenBank/DDBJ under the accession number LC160266.

Results

Identification of native Ps6GT31A

Paenibacillus sp. 598K was cultivated in a medium containing various carbon sources, namely glucose, sucrose, Fujioligo G67, dextrin, Matsunorin M-22 starch, Isomalto 500, pullulan, nigerose, kojibiose, B1299 glucan, B1355 glucan, and dextran 40 (Figure S1), and the CI-producing activity of the secreted enzyme in the culture supernatant against dextran or starch was examined by HPLC, as described previously (Fig. 1) (Funane et al. 2014). At least a trace amount of the CI-producing activity against both dextran and starch was observed in all cultures, and when the bacterium was grown with pullulan, B1299 glucan, B1355 glucan, and dextran 40, the activity clearly increased. The level of CI-producing activity of the pullulan culture was much lower than that of the others. Its growth [optical density (OD) at 600 nm] reached only 0.35 after 3-day cultivation, whereas those of B1299 glucan, B1355 glucan, and dextran 40 cultures reached 1.5–2.0. With other carbohydrates, no significant CI-producing activity was observed (Fig. 1). When the carbon source was dextrin, Matsunorin M-22 starch, or Isomalto 500, bacteria did not grow well at the beginning, the lag phase of which was considerably long (22–35 hours) (Figure S1). CI-producing activities against both dextran and starch were unaffected by glucose addition in dextran 40-supplemented culture broth.

CITase produces CIs from α-(1→6)-consecutive glucose chains of DPs≥4. To make CIs from starch, the bacterium would need to possess the enzyme for elongating α-(1→6)-linkages from starch. We therefore attempted to purify this enzyme. When the crude enzymes, which were pre-purified by ammonium sulfate precipitation from the culture supernatant of the medium containing dextran 40, were subjected to anion chromatography to measure CITase activity against starch (Fig. 2), low CI-producing activity was measured in fractions 38–45 (Fig. 2, solid squares). In contrast, as indicated by the open circles in Fig. 2, high-CI-producing activity against dextran was observed in fractions 45–58, and a shouldered peak was also observed in fractions 38–45, which is comparable to the CI-producing activity against starch. Fractions 45–58 were considered to be CITase because CIs were produced from dextran 40 but not from starch with these fractions. Then, native CITase (fraction 49) was added to the fractions, and CITase activity against starch was remarkably increased at fraction 38-44 (Fig. 2, solid triangles). Among them, the peak fraction 41 reached its CI-producing activity against starch to about 2.4 U/mL. That is approximately the same as the sum of the CI-producing activity against dextran 40 of the shoulder peak and fraction 49 (Fig. 2, open circles). The enzyme eluted at around fraction 41 must produce α-(1→6)-glucose chains from starch usable for CITase. The rest of the fractions except fractions 38–45 shown in Fig. 2 observed no CITase activity against starch even with additional CITase.

The Resource Q column after 0–600 mM NaCl graidient elution was washed further with 1 M NaCl. The eluted proteins did not show any CI-producing activity against starch. Also, the precipitated proteins of 598K culture supernatants with <20% and >60% ammonium sulfate-saturated showed no CITase activity against starch. Therefore, we assumed that the protein eluted at fractions 38–45 was the only enzyme involved in extracellular CI production from starch with CITase in *Paenibacillus* sp. 598K. Fractions 38–45 were thus further purified into a single 135-kDa band on SDS-PAGE (Figure S2, lane 4). The protein in this band exhibited both hydrolytic and transglucosylation activity against maltooligosaccharides, similarly to the 135-kDa protein from *P. agaridevorans* T-3040 (Funane et al. 2014), and it was named Ps6GT31A. Edman analysis of the purified native Ps6GT31A yielded an N-terminal amino acid sequence of AGLGNVTGAVASGDSLTLTLDNG. From the N-terminal amino acid sequence, the *6gt31a* gene encoding Ps6GT31A was found in the *Paenibacillus* sp. 598K genome. The corresponding *Hin*dIII-*Nde*I DNA fragment contained full-length *cit* and *6gt31a* and a partial hypothetical protein-coding gene. The DNA sequence of *6gt31a* was 3846 bp long, and it encoded a 1281-amino-acid protein. BLASTP search of the deduced amino acids indicated that Ps6GT31A has a modular architecture including several functional domains (Fig. 3a). The deduced amino acid sequence of 132 residues was shown to contain a copper amine oxidase-like domain by a BLASTP search.

The two genes, *cit* (106–3024) and *6gt31a* (3084–6929), are closely located in the same orientation in the genome and the partial hypothetical protein-coding gene was observed within positions 7123–7519. The putative promoter regions and terminators were predicted using the Softberry programs BPROM and FindTerm (Solovyev and Salamov 2011). Two possible promoters and one terminator were predicted. The first putative promoter was located upstream of *cit* at position 86 with a −10 box (AATTCAAAT) at position 71 and a −35 box (ATCAAA) at position 46. The second putative promoter was located between *6gt31a* and the partial hypothetical protein gene at position 7060 with a −10 box (TTTTATATT) at position 7045 and a −35 box (CTGAAT) at position 7028. A putative terminator was identified after the stop codon of *6gt31a* at 239 bp downstream from the stop codon of *6gt31a* and also at 194 bp downstream from the palindromic sequence after *6gt31a*.

The deduced amino acid sequence corresponding to the mature Ps6GT31A (residues 36–1281) resembled 6-glucosyltransferase CtsZ from *Bacillus globisporus* C11 (GenBank accession number BAB88404; 61% identity and 75% similarity) (Aga et al. 2002) (Fig. 3). Residues 36–798 exhibited high similarity to the conserved region of GH31 enzymes: The region showed 28% identity (45% similarity) with *Cellvibrio japonicus* α-xylosidase CjXyl31A (Protein Data Bank

[PDB] entry 2XVG) (Larsbrink et al. 2011), 24% identity (42% similarity) with *C. japonicus* oligosaccharide α-1,4-glucosyltransferase CjAgd31B (PDB entry 4B9Y) (Larsbrink et al. 2012), 26% identity (41% similarity) with uncharacterized protein Lmo2446 from *Listeria monocytogenes* (PDB entry 4KMQ), and 23% identity (43% similarity) with α-glucosidase MalA from *Sulfolobus solfataricus* (PDB entry 2G3M) (Ernst et al. 2006). The region including the deduced catalytic domain of Ps6GT31A showed 64% identity (77% similarity) with that of *B. globisporus* CtsZ (Fig. 3b). Residues Asp429 and Asp491 in Ps6GT31A were predicted to be the catalytic nucleophile and the acid/base, respectively. These correspond to Asp433 and Asp495 of *B. globisporus* CtsZ and they are conserved in all of the characterized GH31 enzymes. The NCBI conserved domain search revealed that the enzyme has two family 35 carbohydrate-binding modules (CBM35). In addition, BLASTP search suggested that the remaining C-terminal region is a family 61 carbohydrate-binding module (CBM61). Residues 850–984 and 989–1116, named PsCBM35-1 and PsCBM35-2, respectively, displayed similarities (40% and 37% identity, and 55% and 49% similarity, respectively) to α-(1→6)-glucan-binding module BcCBM35-1 from *P. agaridevorans* T-3040 GH66 CITase (PDB entry 3WNK)(Suzuki et al. 2014). PsCBM35-1 and PsCBM35-2 resembled each other (40% identity, 53% similarity). In addition, residues 1126–1281, named PsCBM61, were 24% identical and 37% similar to β-(1→4)-galactan-binding module TmCBM61 from *Thermotoga maritima* GH53 endo-β-1,4-galactanase (PDB entry 2XOM) (Cid et al. 2010).

Expression and purification of the recombinant protein

The gene encoding mature Ps6GT31A without a secretion signal sequence (residues 1–35) was cloned. Recombinant Ps6GT31A was successfully expressed in *E. coli* and purified as a single band on SDS-PAGE when visualized by staining with Coomassie Brilliant Blue R-250 (Figure S2, lane 3). The molecular mass of the recombinant protein was estimated to be 136 kDa by SDS-PAGE, which was in good agreement with the native Ps6GT31A and the predicted molecular mass (135.6 kDa) from the amino acid sequence.

Substrate specificity of Ps6GT31A

When Ps6GT31A was incubated with starch, glucose was liberated as the product, suggesting that Ps6GT31A has α-glucosidase activity (Fig. 4a). The effects of pH and temperature on this α-glucosidase activity were investigated using starch as the substrate (Figure S3). The enzyme achieved maximal activity at pH 6.0 and 50°C. The enzyme retained more than 80% of its activity between pH 5.5 and pH 8.0 and at temperatures of less than 50°C. Under the optimal reaction conditions (50°C, pH 6.0), the specific activity of Ps6GT31A toward starch was 1.3 units/mg. The activities of the enzyme toward dextran 40 and pullulan were 0.24 and 0.17 units/mg,

respectively. These data suggested that Ps6GT31A exhibits higher hydrolysis activity against α-(1→4)-linked glucan than against α-(1→6)-linked glucan. The enzyme displayed extremely low activity against PNP α-glucopyranoside. Its K_m and k_{cat} values at pH 6.0 at 37°C were 22.2 ± 0.8 mM^{-1} and 0.14 ± 0.01 s^{-1}, respectively. The enzyme did not display any hydrolytic activity against PNP α-mannopyranoside, PNP α-galactopyranoside, and PNP α-xylopyranoside.

The substrate specificity of the enzyme for glucobioses was investigated using G2, kojibiose, nigerose, IG2, trehalose, cellobiose, sophorose, laminaribiose, and gentiobiose as the substrates. The enzyme hydrolyzed α-(1→4)-, α-(1→2)-, α-(1→3)-, and α-(1→6)-linked glucobioses in decreasing order of activity of 100%, 39%, 33%, and 2%, respectively. The enzyme did not hydrolyze trehalose and β-linked glucobioses. In the hydrolysis of panose [α-Glc-(1→6)-α-Glc-(1→4)-Glc], glucose and G2 were generated by Ps6GT31A at the initial stage of the reaction (Fig. 4b). These results revealed that the enzyme liberates glucose from the non-reducing end of the substrate. The k_{cat}/K_m values of Ps6GT31A for the hydrolysis of G2–G7 were 2.3 ± 0.0, 5.6 ± 1.0, 21 ± 3, 40 ± 2, 45 ± 9, and 39 ± 9 $s^{-1}\mu M^{-1}$, respectively. The catalytic efficiency increased as the DP increased from 2 to 5 and remained at the same level at DPs of 5–7, indicating that Ps6GT31A recognizes substrates with lengths comparable to that of G5.

Transglucosylation activity of Ps6GT31A

When Ps6GT31A was incubated with 1% (w/v) G2–G7 or 1% (w/v) IG2–IG7 as the substrates, it produced longer oligosaccharides than each substrate besides degradation products of glucose and short oligosaccharides, indicating that Ps6GT31A also had transglucosylation activity (Figs. 5a and 5b). The enzyme also catalyzed the transglucosylation of kojibiose and nigerose but not trehalose (Fig. 5c). These results indicated that Ps6GT31A had transglucosylation activity toward α-(1→2)-, α-(1→3)-, α-(1→4)-, and α-(1→6)-linked glucobioses. The transglucosylation/hydrolysis ratios of Ps6GT31A were calculated from the amounts of hydrolytic products, substrate, and transglucosylation products using maltooligosaccharides as the substrates. The transglucosylation/hydrolysis ratios of the enzyme for G2, G3, G4, and G5 were as follows: 5:95, 7:93, 21:79, and 18:82, respectively. The results indicated that maltooligosaccharides with DPs ≥4 were good acceptors of Ps6GT31A relative to short maltooligosaccharides.

The hydrolytic and transglucosylation products of G4 and IG4 produced by the enzyme at the initial stage were analyzed by HPLC-ELSD (Fig. 6). When G4 was used as the substrate, a new larger product with a longer retention time than G4, which was considered a transglucosylation product as denoted by an asterisk in Fig. 6a, was detected in addition to hydrolytic products (glucose and G3). Similarly, the hydrolytic products (glucose and IG3) and the transglucosylation product IG5 were detected when IG4 was used for a substrate (Fig. 6b).

Because the transglucosylated product from IG4 was IG5, the new product from G4 should be a transglucosylation product of G4 attached via α-(1→6)-linked glucose. To identify the transglucosylation products of G4, FT-MS and ^{13}C NMR analyses were performed (Figure S4). FT-MS analysis gave rise to one $[M-H]^-$ ion at *m*/*z* 827.3, corresponding to a glucosyl oligosaccharide with a DP of 5 (Figure S4a). The product had ^{13}C NMR signals (D_2O, δ in ppm) of 100.8 [C1 of non-reducing end α-Glc-(1→6)-] and 68.6 [C6 of -α-(1→6)-Glc-α-(1→4)-] [Figure S4b(6)], which are never observed in maltopentaose [Figure S4b(5)]. These results revealed that the enzyme transferred one glucose to the non-reducing end of substrate G4 and elongated oligosaccharides with α-(1→6)-linkages.

Transglucosylation products by Ps6GT31A and CI production by CITase

Ten fractions of hydrolysis and transglucosylation reaction products from G5 after 24-h Ps6GT31A treatment were obtained by HPLC separation and named fractions 1–10 (Fig. 7a). Fractions 1–3 were identified as glucose, G2, and G3, respectively, on the basis of the retention times. FT-MS analysis of fractions 2–10 revealed $[M+H]^+$ ions at *m*/*z* 343.1, 505.1, 667.2, 829.3, 991.2, 1153.4, 1315.5, 1477.7, and 1639.8, corresponding to glucooligosaccharides with DP2–10 (Table 1), respectively. To examine whether CITase produces CI from these products, fractions 4–10 were individually incubated with CITase. Then the reaction mixture was incubated with glucoamylase and highly branched dextran hydrolase. The remaining linear oligosaccharides are digested completely to glucose with this treatment, and intact cyclic oligosaccharides are left. When fractions 4 and 5 were used as the substrates, CI production was not detected. By contrast, CI production was detected when CITase was incubated with fractions 6–10 (Table 1). Figure 7b shows CI production by CITase when the enzyme was incubated with fraction 7 as the substrate. CITase mainly produced CI-7–9 from fraction 7. When fractions 6–10 were used as the substrates, the amounts of CI produced by CITase, which were the sums of CI-7–9, were 53.7 ± 0.1, 69.0 ± 4.4, 139.6 ± 2.7, 122.5 ± 4.3, and 197.8 ± 6.0 μM (Table 1), respectively. CITase produced larger amounts of CI when larger molecules of fractions 8–10 were used as the substrates. The results indicated that CITase can utilize the Ps6GT31A-transglucosylation products from maltooligosaccharides as substrates for producing CIs.

Fractions 4–10 were individually treated with oligo-1,6-glucosidase and/or *B. stearothermophilus* α-glucosidase and subsequently analyzed by HPLC-ELSD. Oligo-1,6-glucosidase specifically hydrolyzes the non-reducing end of α-(1→6)-glucosidic linkages of isomaltooligosaccharides. By contrast, *B. stearothermophilus* α-glucosidase specifically hydrolyzes α-(1→4)-glucosidic linkages from the non-reducing end of oligosaccharides. When fraction 7 was treated with oligo-1,6-glucosidase, glucose, G4, G5, and two additional peaks were

detected by HPLC (Fig. 7c). This indicated that fraction 7 consisted of a mixture of two major heptasaccharides, α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{IV}G4 and α-Glc-(1→6)-α-Glc-(1→6)-^{V}G5 (Table 1, Roman numerals indicate the substituted residues in ascending order starting from the reducing end). When fraction 7 was treated with a mixture of oligo-1,6-glucosidase and *B. stearothermophilus* α-glucosidase, it was almost completely hydrolyzed to glucose (Fig. 7c, bottom panel). Similarly, fractions 4–10 were also completely hydrolyzed to glucose by these enzymes (data not shown). These results indicated that fractions 4–10 contained only α-(1→6)- and/or α-(1→4)-linked glucoses. The structures and ratios of the major reaction products estimated by enzymatic treatment and the amount of CI produced by CITase when they were used as substrates are shown in Table 1. The results of oligo-1,6-glucosidase treatment indicated that Ps6GT31A created α-(1→6)-linked glucosyl moieties at the non-reducing end of maltooligosaccharide through successive transglucosylation reactions. Actually, all transglucosylation products isolated in fractions 4–10 are such types of glucooligosacharide. CI production by CITase was observed in the case of fractions 6–10 but not fraction 4 or 5. The amounts of CIs produced from fractions 8–10 were greater than those produced from fractions 6 and 7.

Discussion

Glycoside hydrolases and carbohydrate-binding modules are classified into families in the Carbohydrate-Active EnZymes (CAZy) database according to the similarity between their amino acid sequences (Lombard et al. 2013). GH31 includes α-glucosidase (EC 3.2.1.20), α-1,3-glucosidase (EC 3.2.1.84), sucrase-isomaltase (EC 3.2.1.48, EC 3.2.1.10), and α-xylosidase (EC 3.2.1.177). It also includes some transferases involved in the rearrangement of α-glucans, such as cycloalternan synthetic enzymes CtsY and CtsZ from *Bacillus* sp. (Kim et al. 2003; Nishimoto et al. 2002) and the glycogen synthetic enzyme oligosaccharide α-1,4-glucosyltransferase from *C. japonicus* (Larsbrink et al. 2012). GH31 enzymes act with a retaining mechanism; therefore, GH31 includes α-glucosidases with strong transglucosylation activity (Kato et al. 2002; Ota et al. 2009).

Ps6GT31A was isolated from *Paenibacillus* sp. 598K culture supernatant as the only enzyme that produces a substrate for CITase to synthesize CIs using starch or maltooligosaccaharides. It was revealed to be a GH31 enzyme with broad-specificity α-glucosidase activity and strong α-(1→6)-transglucosylation activity. The enzyme transferred one glucosyl residue from the non-reducing end of maltooligosaccharide to the non-reducing end of another molecule to produce an α-(1→6)-glucosyl linkage. The α-(1→6)-glucosyl moiety was then elongated by the successive addition of glucose to the non-reducing end of the growing chain via the transglucosylation activity of the enzyme in these fractions. The reaction products from G5 generated by Ps6GT31A summarized in Table 1 indicate that fraction 4 includes α-Glc-(1→6)-^{III}G3 and that fraction 5 includes α-Glc-(1→6)-^{IV}G4 and α-Glc-(1→6)-α-Glc-(1→6)-^{III}G3. These results suggest that the α-(1→6)-glucosyl moiety with DP 2–3 was introduced at the non-reducing end of the oligosaccharide via successive transglucosylation of the enzyme. The results for oligo-1,6-glucosidase-treated fractions 6 and 7 indicate that the enzyme produced hexasaccharides containing α-(1→6)-glucosyl moieties with DP 2–4 and heptasaccharides containing α-(1→6)-glucosyl moieties with DP 3–4. In the case of fractions 8–10, oligosaccharides containing α-(1→6)-glucosyl moieties with DP of ≥5 were found. When CITase was incubated with fractions 4–10 as the substrates, CI production was observed for fractions 6–10 but not for fraction 4 or 5. These results indicate that the IG4 component at the non-reducing end of the substrate was necessary for CI production, which is supported by the previous finding that IG4 is the smallest substrate of CITase for CI production (Suzuki et al. 2012). Because fractions 8–10 contained oligosaccharides with longer α-(1→6)-glucosyl chains than IG4, fractions 8–10 are likely to be better substrates for CITase than fractions 6 and 7, resulting in higher levels of CI production.

The deduced amino acid sequence of mature Ps6GT31A resembled 6-

glucosyltransferase from *B. globisporus* C11, which is involved in cycloalternan synthesis (Nishimoto et al. 2002). The enzyme catalyzes the α-(1→6)-transglucosylation of one glucosyl residue to the non-reducing end of maltooligosaccharide to produce α-Glc-(1→6)-maltooligosaccharide, but not the successive α-(1→6)-transglucosylation to produce the α-(1→6)-glucosyl chain. From the perspective of the enzymatic action of α-(1→6)-transglucosylation, this enzyme is similar to Ps6GT31A, but Ps6GT31A was distinctly different from *B. globisporus* 6-glucosyltransferase in terms of the number of transglucosylated glucose residues.

A BLAST search revealed that Ps6GT31A has at least three CBMs, PsCBM35-1, CBM35-2, and PsCBM61, on its C-terminal side (Fig. 3a). The deduced amino acid sequences of PsCBM35-1 and PsCBM35-2 resembled the α-(1→6)-glucan-binding module BcCBM35-1 of *P. agaridevorans* T-3040 CITase (Fig. 3c). Aromatic residues such as tryptophan and histidine, which bind to glucose at the BcCBM35-1 sugar-binding site, were conserved in PsCBM35-1 and PsCBM35-2 (His875, Trp943, His1009, and Trp1077) of Ps6GT31A (Suzuki et al. 2014). These aromatic residues were also conserved in CBMs of 6-glucosyltransferase (Fig. 3c). There are four different kinds of CBM35, which show affinity toward α-glucan, mannan, xylan, or β-1,3-galactan (Bolam et al. 2004; Ichinose et al. 2005; Suzuki et al. 2015). PsCBM35-1 and PsCBM35-2 would be expected to be members of the α-glucan-binding subfamily. The C-terminal PsCBM61 displayed similarity with β-(1→4)-galactan-binding module TmCBM61 from *T. maritima* endo-β-1,4-galactanase (Cid et al. 2010) (Fig. 3d). TmCBM61 has three tryptophans in the β-(1→4)-galactan-binding site. Typ508 in TmCBM61 was replaced by histidine in Ps6GT31A (His1181) and phenylalanine in 6-glucosyltransferase (Phe1185). The other residues were not conserved in Ps6GT31A and 6-glucosyltransferase. Although further analysis of Ps6GT31A will be necessary to understand α-glucan recognition, there are some distinct differences in sugar-binding sites between α-glucan-binding and β-1,3-glucan-binding CBM61s.

In this paper, we describe that *Paenibacillus* sp. 598K produced CI without dextran via the contribution of Ps6GT31A. The *6gt31a* gene is located immediately downstream from the *cit* gene in the *Paenibacillus* sp. 598K genome and there are no promoter or palindromic sequences between these genes. The *6gt31a* gene and the downstream gene are at the distance of 241 bp from each other and are dissociable by a palindromic sequence. Therefore, only *cit* and *6gt31a* should be regulated by the same promoter and co-expressed. The CITase activity of the bacterium was induced by pullulan, B1299 glucan, B1355 glucan, and dextran, but not by other sugars including isomaltooligosaccharides. The α-glucan containing an α-(1→6)-linkage should be necessary, but the short DP of α-(1→6)-linked glucoses does not seem to be sufficient for CI

production in *Paenibacillus* sp. 598K. Glucose addition in dextran 40-supplemented culture broth had no effect on CITase activity. In the case of *P. agaridevorans* T-3040, activity of the 135-kDa protein (most probably 6GT31A) was induced by starch and dextran, and suppressed by the addition of glucose to the culture broth (Funane et al. 2014). Conversely, its CITase was reported to be induced by starch, dextran, and even by small molecules of isomaltooligosaccharides of DP = 2–3, and the addition of glucose did not affect CITase production upon growth with dextran or isomaltooligosaccharides, but it was almost completely inhibited upon growth with starch. The *cit* and *6gt31a* genes are likely to be expressed simultaneously and regulated in the same way in *Paenibacillus* sp. 598K, whereas these genes are considered to be differently expressed and regulated in *P. agaridevorans* T-3040. Despite these differences, the growth patterns of *P. agaridevorans* T-3040 and *Paenibacillus* sp. 598K showed some similarity. These bacterial strains grow well with dextran as the sole carbon source but less well with starch, showing a long lag phase of around 1 day (Figure S1) (Funane et al. 2014). It seems that dextran is a good carbon source for these CI-producing bacteria for both their growth and CI production, but dextran is usually produced by other bacteria such as dextransucrase-producing lactic acid bacteria or dextran dextrinase-producing acetic acid bacteria, as mentioned previously. While CI-producing bacteria use dextran as a carbon source, they induce another CI-producing enzymatic system, which enables them to produce CIs from starch. One possible function of CIs for these bacteria is as their exclusive carbon source for nutritional purposes. They may have multiple ways of producing CIs from different materials for their survival, but further investigations will be required to understand the meaning of CI production for the bacterial strains.

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Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

This article does not describe any studies on human participants or animals performed by any of the authors.

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Figure legends

Fig. 1 CI-producing activity against dextran and starch in the culture supernatants of a medium containing various carbon sources

White bar, CITase activity with 2% (w/v) dextran 40; black bar, CITase activity with 2% (w/v) starch. The error bars indicate the standard deviation of triplicate experiments. None, no carbon source; Mat M-22 starch, Matsunorin M-22 starch; B1299 glucan, *L. mesenteroides* NRRL B-1299 glucan; B1355 glucan, *L. mesenteroides* NRRL B-1355 glucan; Dextran 40+Glc, 2% (w/v) dextran 40 with 1% (w/v) glucose.

Fig. 2 Purification of Ps6GT31A by Resource Q chromatography

CITase activities against dextran 40 and starch in each fraction were measured as described previously (Suzuki et al. 2012). White circle, CITase activity when dextran 40 was used as the substrate; solid square, CI-producing activity when starch was used as the substrate; solid triangle, CI-producing activity when starch was used as the substrate, supplemented with fraction no. 49 (native CITase) added to the fractions at a 1:1 ratio (v/v).

Fig.3 Primary structure of Ps6GT31A

(a) Schematic drawing of the Ps6GT31A molecular architecture. GH31 conserved region, highly conserved region in glycoside hydrolase family 31; PsCBM35-1 and PsCBM35-2, carbohydrate-binding module family 35; PsCBM61, carbohydrate-binding module family 61. The numbers above the bars denote the amino acid residue numbers. (b) Partial sequence comparison with Ps6GT31A and *B. globisporus*C11 6-glucosyltransferase (Bg6GT) (Aga et al. 2002). The alignment was performed using ClustalW2 (Larkin et al. 2007). Identical amino acid residues are in black boxes and similar residues in gray boxes. The asterisks indicate the putative catalytic residues. (c) Sequence alignment of CBM35 among PsCBM35-1, PsCBM35-2, *B. globisporus* C11 6-glucosyltransferase BgCBM35-1, BgCBM35-2, and *Paenibacillus agaridevorans* CITase BcCBM35-1 (Uniprot ID P94286) (Suzuki et al. 2014). The residues corresponding to BcCBM35-1 sugar-binding sites are boxed. (d) Sequence alignment of CBM61s among PsCBM61, 6-glucosyltransferase BgCBM61, and *T. maritima* endo-β-1,4-galactanase TmCBM61 (Uniprot ID Q9X0S8) (Cid et al. 2010). Open circles (○) indicate residues consisting of TmCBM61 sugar-binding sites.

Fig. 4 HPLC-ELSD analysis of the hydrolyzed products of (a) starch and (b) panose generated by Ps6GT31A

Ps6GT31A (120 nM) was incubated with 0.5% (w/v) starch in 30 mM Tris-malate buffer (pH 6.0) at 50°C. For panose, the enzyme was incubated with 0.1 mM substrate in 40 mM Tris-malate buffer (pH 6.0) at 50°C. Reaction products were analyzed by HPLC-ELSD. An acetonitrile–water (60:40, v/v) mixture was used as a mobile phase. Abbreviations: Glc, glucose; G2, maltose; Pa, panose.

Fig. 5 TLC analysis of the reaction products of glucooligosaccharides generated by Ps6GT31A

(a) S1, glucose and G2–G7 (standards); 1, glucose used as the substrate; 2, G2 used as the substrate; 3, G3 used as the substrate; 4, G4 used as the substrate: 5, G5 used as the substrate; 6, G6 used as the substrate; 7, G7 used as the substrate. (b) S2, IG2–IG7 (standards); 1, glucose used as the substrate; 2, IG2 used as the substrate; 3, IG3 used as the substrate; 4, IG4 used as the substrate; 5, IG5 used as the substrate; 6, IG6 used as the substrate; 7, IG7 used as the substrate; E, enzyme only. (c) S3, glucose (standard); T, trehalose used as the substrate; K, kojibiose used as the substrate; N, nigerose used as the substrate; –, no enzyme; +, incubated with the enzyme. Ps6GT31A (350 nM) was incubated with 1% (w/v) substrate in 40 mM Tris-malate buffer (pH 6.0) at 50°C for 24 h. The products were examined by TLC using silica gel 60 F_{254} in a solvent system of 1-butanol:acetic acid:water at a ratio of 2:1:1 (a and b) or acetonitrile:water at a ratio of 4:1 (c).

Abbreviations: Glc, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose; IG2, isomaltose; IG3, isomaltotriose; IG4, isomaltotetraose; IG5, isomaltopentaose; IG6, isomaltohexaose; IG7, isomaltoheptaose.

Fig. 6 HPLC-ELSD analysis of the reaction products of (a) maltotetraose and (b) isomaltotetraose produced by Ps6GT31A

Reaction products of maltotetraose (a) and isomaltotetraose (b) at the initial stage were analyzed. The new product generated by the enzyme is indicated by an asterisk.

Abbreviations: Glc, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; IG2, isomaltose; IG3, isomaltotriose; IG4, isomaltotetraose; IG5, isomaltopentaose. An acetonitrile–water (60:40, v/v) mixture was used as the mobile phase.

Fig. 7 HPLC-ELSD analysis of the reaction products of maltopentaose produced by Ps6GT31A and the hydrolytic products produced by glucosidases

(a) Reaction products of maltopentaose produced by Ps6GT31A. Ten fractions were named fractions1–10. (b) CI production by CITase using fraction 7 as the substrate. Fraction 7 was

incubated with (+) or without (−) CITase. The reaction products were digested with glucoamylase and highly branched dextran hydrolase, and they were analyzed by HPLC-ELSD as described previously (Funane et al. 2014). (c) Enzymatic hydrolysis of fraction 7. Top panel, Standards (glucose and maltooligosaccharides); second panel, fraction 7 (untreated); third panel, oligo-1,6-glucosidase-treated (/α-1,6); bottom panel, oligo-1,6-glucosidase and *B. stearothermophilus* α-glucosidase-treated (/α-1,6/α-1,4). Abbreviations: Glc, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose.

Table 1

Reaction products of G5 produced by Ps6GT31A as indicated in Figure 7a and CI production from the enzyme product by CITase

Fraction number	Observed *m/z* $[M+H]^+$	DP	[a]Major products (%)	[b]CI produced (μM)
2	343.1	2	G2 (100)	Not determined
3	505.1	3	G3 (100)	Not determined
4	667.2	4	α-Glc-(1→6)-^{III}G3 (19) G4 (13)	Not detected
5	829.3	5	α-Glc-(1→6)-α-Glc-(1→6)-^{III}G3 (16) α-Glc-(1→6)-^{IV}G4 (23) G5 (4)	Not detected
6	991.2	6	α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{III}G3 (4) α-Glc-(1→6)-α-Glc-(1→6)-^{IV}G4 (55) α-Glc-(1→6)-^{V}G5 (13)	53.7 ± 0.1
7	1153.4	7	α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{IV}G4 (17) α-Glc-(1→6)-α-Glc-(1→6)-^{V}G5 (49)	69.0 ± 4.4
8	1315.5	8	α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{IV}G4 (14) α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{V}G5 (23)	139.6 ± 2.7
9	1477.7	9	α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{IV}G4 (11) α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-^{V}G5 (25)	122.5 ± 4.3
10	1639.8	10	α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α- (9)	197.8 ± 6.0

			Glc-(1→6)-^{IV}G4 (20) α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)-α-Glc-(1→6)- ^{V}G5	

[a]The value is the average of two independent assays. [b]Results are presented as the mean ± S.E. The assay was performed in triplicate.

Roman numerals indicate the substituted residues in ascending order stating from the reducing end.

Figure

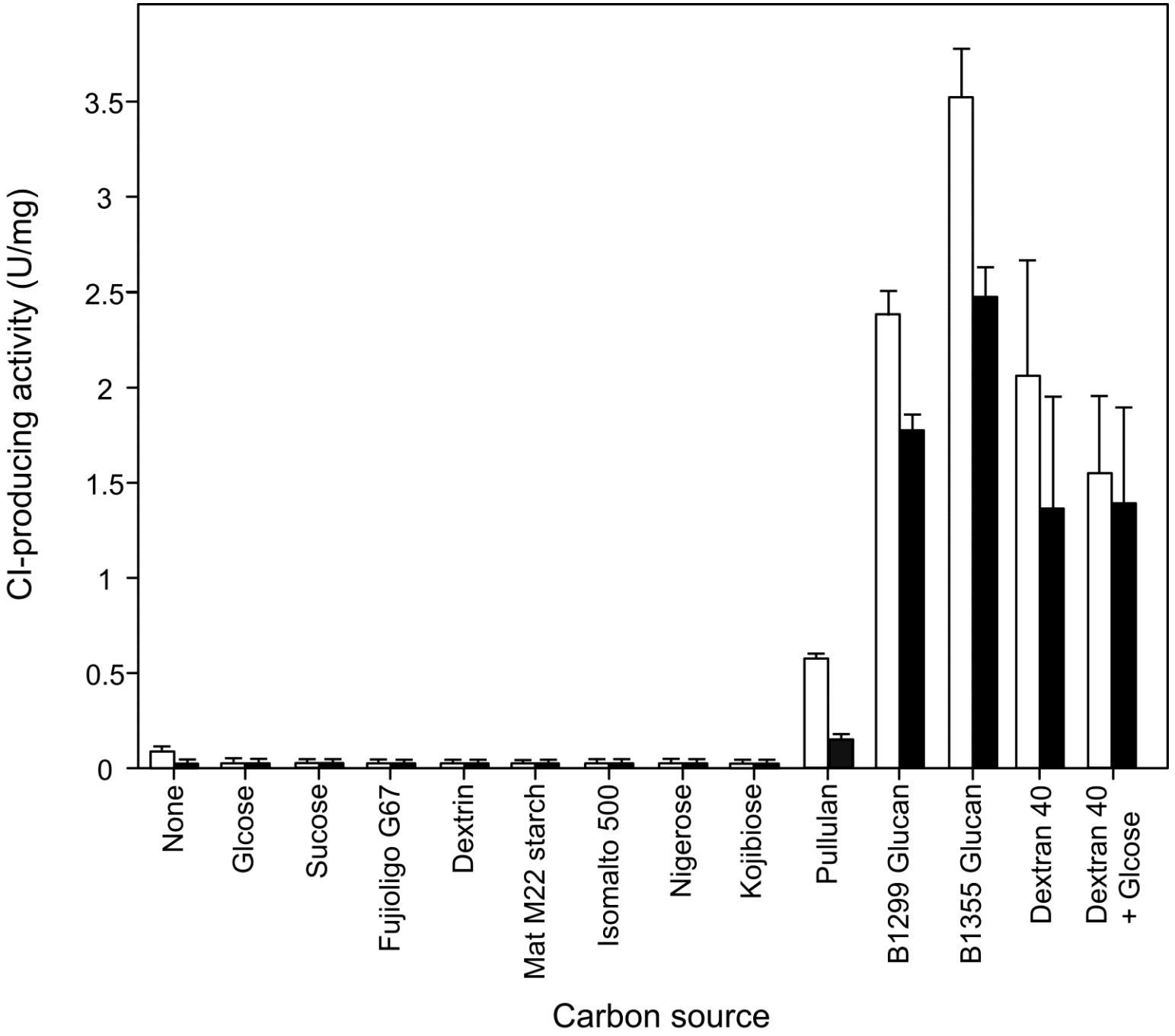

CI-producing activity (U/mg)
0
0.5
1
1.5
2
2.5
3
3.5
None
Glcose
Sucose
Fujioligo G67
Dextrin
Mat M22 starch
Isomalto 500
Nigerose
Kojibiose
Pullulan
B1299 Glucan
B1355 Glucan
Dextran 40
Dextran 40 + Glcose
Carbon source

Fig. 1

Figure

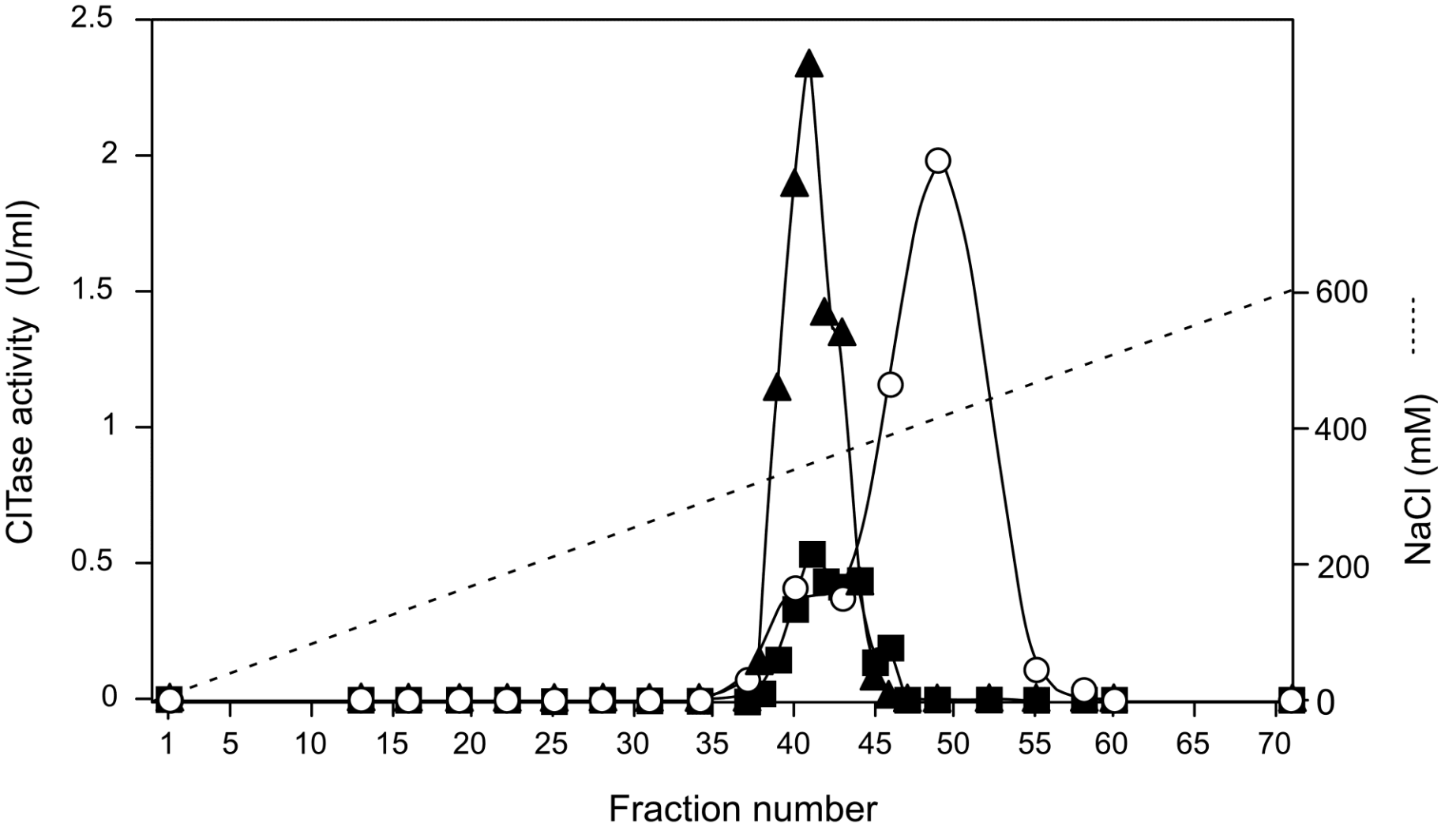
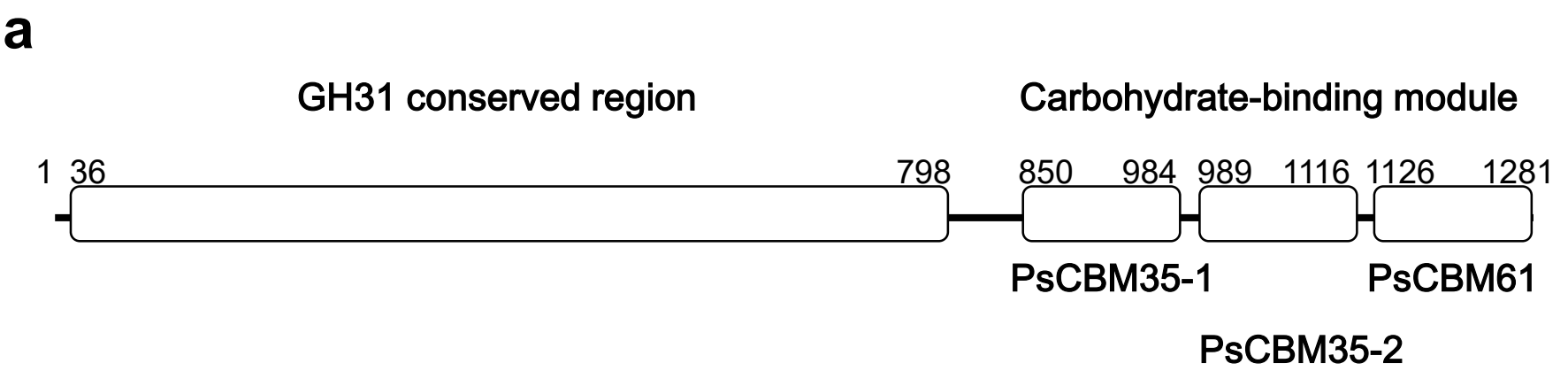

2.5
2
1.5
1
0.5
0
ClTase activity (U/ml)
600
400
200
0
NaCl (mM)
1
5
10
15
20
25
30
35
40
45
50
55
60
65
70
Fraction number

Fig. 2

Figure



b

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Ps6GT31A    36 --AGLGNVTGAVASGDSLTLTLDNGTSAS-DILELDVLSEELLRVDYRPSGAAPSPSTPM  92
Bg6GT       36 YVSSLGNLISSSVTGDTLTLTVDNGAEPSDDLLIVQAVQNGILKVDYRPNSITPSAKTPM  95

Ps6GT31A    93 IDPDASWDAVGATIDTSGDPIVVTTPRMRIEIARTPARMTIKKADGTTLLWEPASGGVFE 152
Bg6GT       96 LDPNKTWSAVGATINTTANPMTITTSNMKIEITKNPVRMTVKKADGTTLFWEPSGGGVFS 155

Ps6GT31A   153 DGVRFQRGSTDNIYGIRSFNAQEDVGGLLRNSSDHPAHAGQQGDAGGPFMWSTAGYGVLV 212
Bg6GT      156 DGVRFLHATGDNMYGIRSFNAFDSGGDLLRNSSNHAAHAGEQGDSGGPLIWSTAGYGLLV 215

Ps6GT31A   213 DSDGGYPYTD-TTGKLEFYYGGTPTEGRRYTKTNVEYYIMVGEPKEIMASYAQVTGTAPM 271
Bg6GT      216 DSDGGYPYTDSTTGQMEFYYGGTPPEGRRYAKQNVEYYIMLGTPKEIMTDVGEITGKPPM 275

Ps6GT31A   272 LPKWSLGFMNFEWGIDQDELEAHVDGYRARNIPIDAFALDYDWMDYGEDNYGEFRWNTDN 331
Bg6GT      276 LPKWSLGFMNFEWDTNQTEFTNNVDTYRAKNIPIDAYAFDYDWKKYGETNYGEFAWNTTN 335

Ps6GT31A   332 FPDAATTQLKEDMEAEGIRLIGIRKPRIITRDFANQRTQQYYDADSNGYFYPGHNEYTDY 391
Bg6GT      336 FPSASTTSLKSTMDAKGIKMIGITKPRIVTKDASANVTTQGTDATNGGYFYPGHNEYQDY 395

Ps6GT31A   392 FIPVTVRSFDPYQQASRDWWQHSIDAFDKGIVGWWNDETDKVDSGSAQYWFGNFSTGFT 451
Bg6GT      396 FIPVTVRSIDPYNANERAWFWNHSTDALNKGIVGWWNDETDKVSSGGALYWFGNFTTGHM 455

Ps6GT31A   452 SQAMYDGQRDYTNDGVRVWQTARSYYPGAQRYATTLWSGDIGTQFYKGELFNWAPGMQEQ 511
Bg6GT      456 SQTMYEGGRAYTSGAQRVWQTARTFYPGAQRYATTLWSGDIGIQYNKGERINWAAGMQEQ 515

Ps6GT31A   512 PRIMLSSANLGQPKWGMDTGGFNSLGGASGPNPSPELYTRWMQFGAFTPVFRVHGNYNQQ 571
Bg6GT      516 RAVMLSSVNNGQVKWGMDTGGFNQQDGTTN-NPNPDLYARWMQFSALTPVFRVHGNNHQQ 574

Ps6GT31A   572 RQPWLYGATAEEASKAVMHTRYSLLPYMYAYEREASETGLGLIKPLLFDYPNDPQAADYT 631
Bg6GT      575 RQPWYFGSTAEEASKEAIQLRYSLIPYMYAYERSAYENGNGLVRPLMQAYPTDAAVKNYT 634

Ps6GT31A   632 EAWMFGDWLLVSPVLGEAQHSKQIYLPAGTWIDYHRGQTYSGGQTIHYPVNADTWTDVPL 691
Bg6GT      635 DAWMFGDWLLAAPVVDKQQTSKDIYLPSGSWIDYARGNAITGGQTIRYSVNPDTLTDMPL 694

Ps6GT31A   692 FVKQGAIIPNQQVLDYVDQQSVTIVNVDIFPSASETSFTYYEDDGSSYDYESGSSFEQRL 751
Bg6GT      695 FIKKGAIIPTQKVQDYVGQASVTSVDVDVFPDTTQSSFTYYDDDGASYNYESGTYFKQNM 754

Ps6GT31A   752 AAQDLSS-SVRVEVGAGSGSYTPDVQHYVLKIHGRAGSAVTAGGSALT 798
Bg6GT      755 TAQDNGSGSLSFTLGAKSGSYTPALQSYIVKLHGSAGTSVTNNSAAMT 802
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c

```
PsCBM35-1   852 AIYEVEDASRSGATPTTRAGINTNHSGYSGSGFVDKLDVPGAAVTVYANAPVSGDYPVEL  911
BgCBM35-1   856 SQYEAEDASLSGNSVAAKASINTNHTGYTGTGFVDGLGNDGAGVTFYPKVKTGGDYNVSL  915
PsCBM35-2   990 AEYAAESAKLWGG-----AGTSQDHWFYKGAAFVDNLTGVGAEASFDVYAPSAGTYNLSL 1044
BgCBM35-2   994 GKYEAESAELSGG-----SSLNTNHWYYSGTAFVDGLSAVGAQVKYNVNVPSAGSYQVAL 1048
BcCBM35-1   421 TRYEAESASMTNV------STNTNHAGYTGSGFVDQFASTGDKVSFAINAPEAGDYSLVF  474

PsCBM35-1   912 RYANGSGSAKTLSVYVNAARVQQLSLADTG-AWSQWGTQT-TTLPLTAGQNIITYKYDSD  969
BgCBM35-1   916 RYANASGTAKSVSIFVNGKRVKSTSLANLA-NWDTWSTQS-ETLPLTAGVNVVTYKYYSD  973
PsCBM35-2  1045 RYANGTGSTKTLSAIVNGGAASTVTLTSPGMNWNLWNEHT-MTATLTAGRNTISFRR--N 1101
BgCBM35-2  1049 RYANGSAATKTLSTYINGAKLGQTSFTSPGTNWNVWQDNV-QTVTLNAGANTIAFKY--D 1105
BcCBM35-1   475 RYGNNTGANSTLNLYVDGNFVQKLYFFNQS-SWGTWKHDAWYQVPLTQGAHTVELRY--E  531

PsCBM35-1   970 AGDTGGVNLDYIRV  983
BgCBM35-1   974 AGDTGNVNIDNITV  987
PsCBM35-2  1102 SGNSGNVNLDRLAV 1115
BgCBM35-2  1106 AADSGNINVDRLLL 1119
BcCBM35-1   532 SGNVGAVNLDSLTL  545
```

d

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PsCBM61    1126 ERNLLDNGDFERDTTYNSNWTQWQPSGQPSAFGIDSGNALHPPEGPARRNQRAYFHSDNA 1185
BgCBM61    1130 EQNLLDNPGFERDTSQTNNWIEWHPGTQAVAFGVDSGSTTNPPESPWSGDKRAYFFAAGA 1189
TmCBM61     461 SRNYLKNPGFETG-----EFSPWRVSGDKKAVKVVKAN---PSSNAHQGEYAVNEWLDES  512

PsCBM61    1186 YQQSIHQVVDVPVNNATYRLEAKVRMKNTTPTTARAEVQGHGGSPIYANISNDG--VWKT 1243
BgCBM61    1190 YQQSIHQTISVPVNNVKYKFEAWVRMKNTTPTTARAEIQNYGGSAIYANISNSG--VWKY 1246
TmCBM61     513 FSFELSQEVELPAG--VYRVGFWTHGE--KGVKIALKVSDYGGNERSVEVETTGWLEWKN  568

PsCBM61    1244 IVIDNINVTSGSVDVGFYVDSPGYTTLHIDEVTLTRAP 1281
BgCBM61    1247 ISVSDIMVTNGQIDVGFYVDSPGGTTLHIDDVRVTKQ  1284
TmCBM61     569 PEIRNIKVETGRIKITVSVEGRAGDWGFIDDFYLFREE  606
```

Fig. 3

Figure

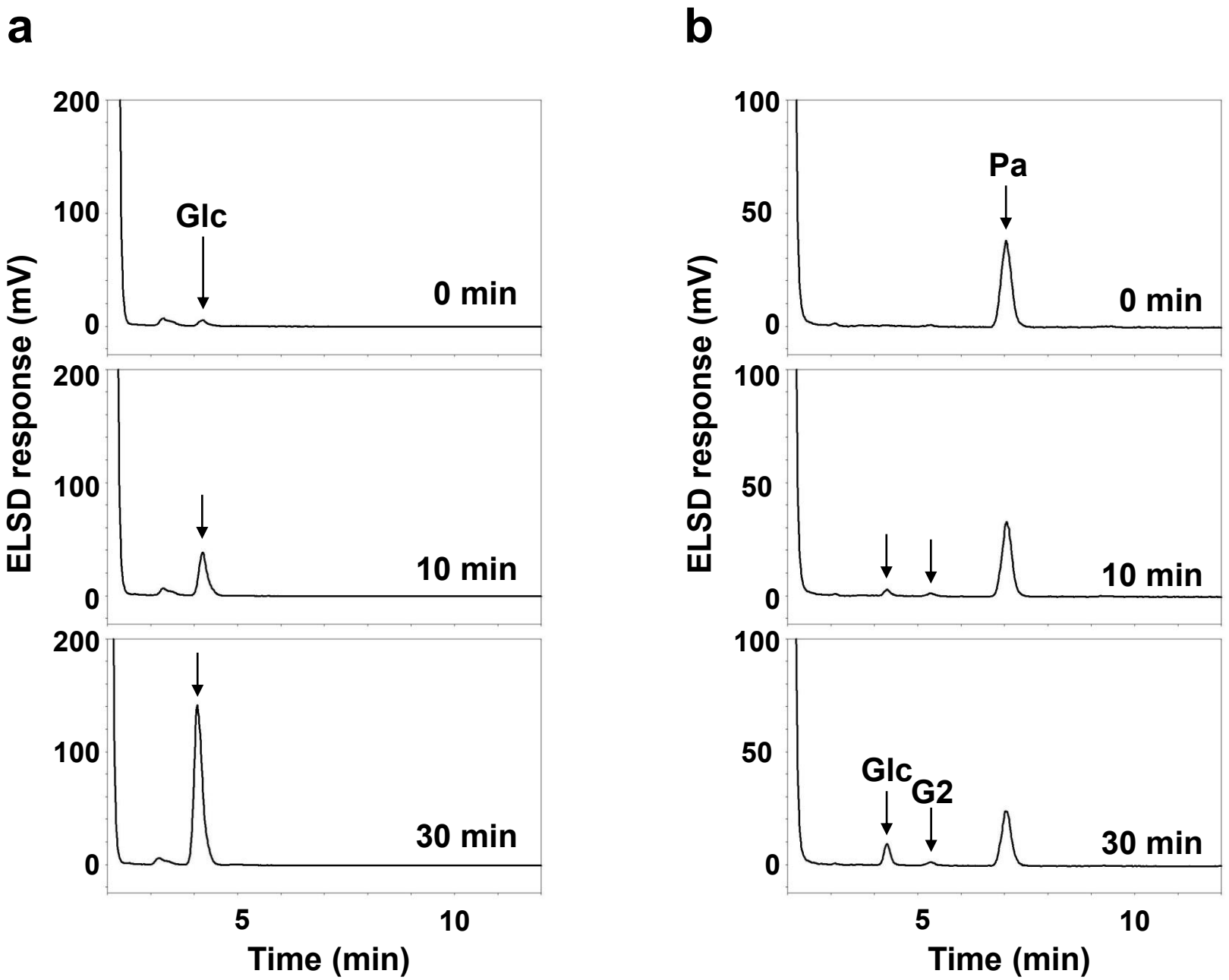
a
200
100
0
Glc
0 min
200
100
0
10 min
200
100
0
30 min
ELSD response (mV)
5
10
Time (min)
b
100
50
0
Pa
0 min
100
50
0
10 min
100
50
0
Glc
G2
30 min
ELSD response (mV)
5
10
Time (min)

Fig. 4

Figure

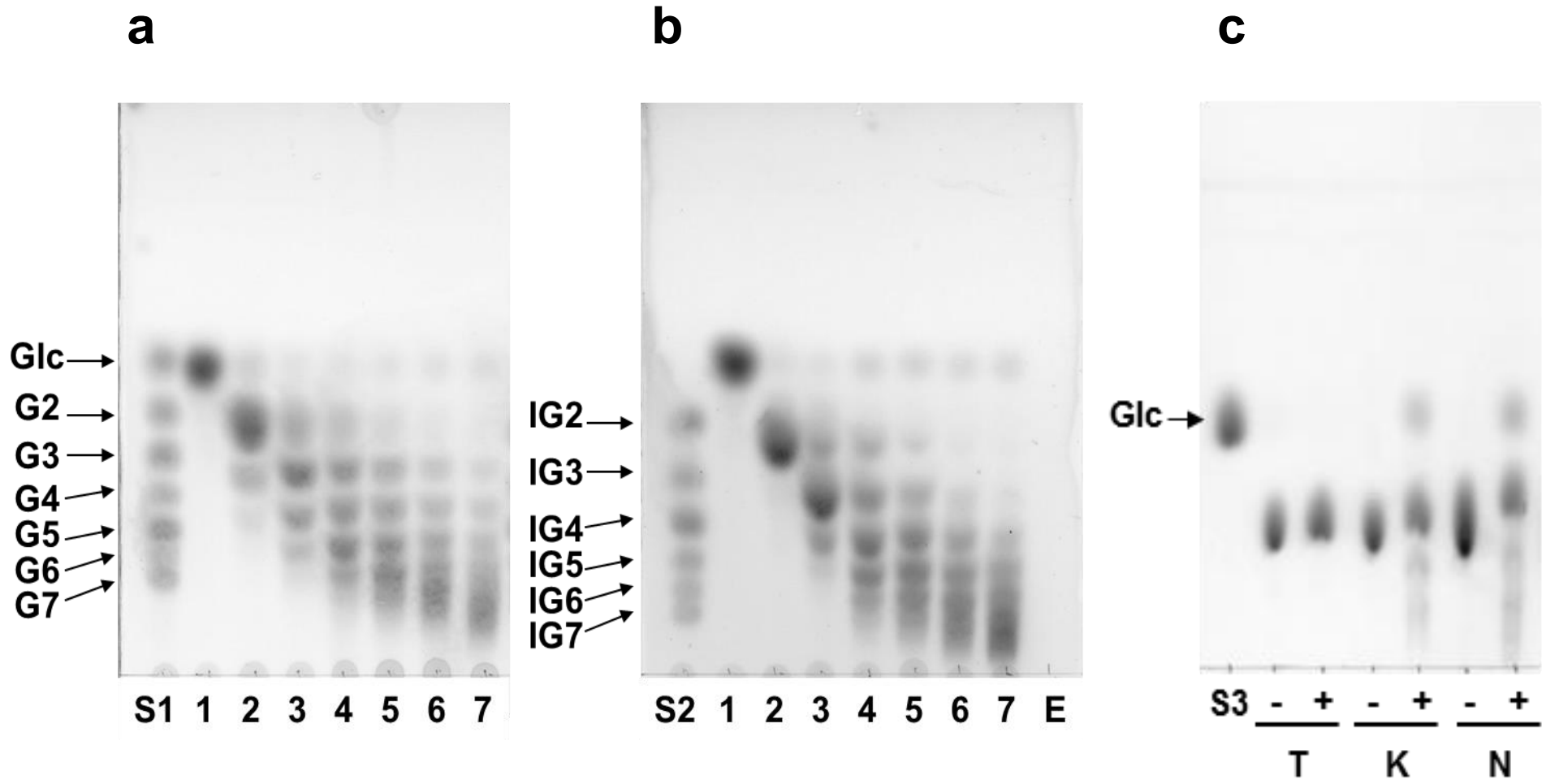
a
Glc
G2
G3
G4
G5
G6
G7
S1 1 2 3 4 5 6 7
b
IG2
IG3
IG4
IG5
IG6
IG7
S2 1 2 3 4 5 6 7 E
c
Glc
S3 - + - + - +
T K N

Fig. 5

Figure

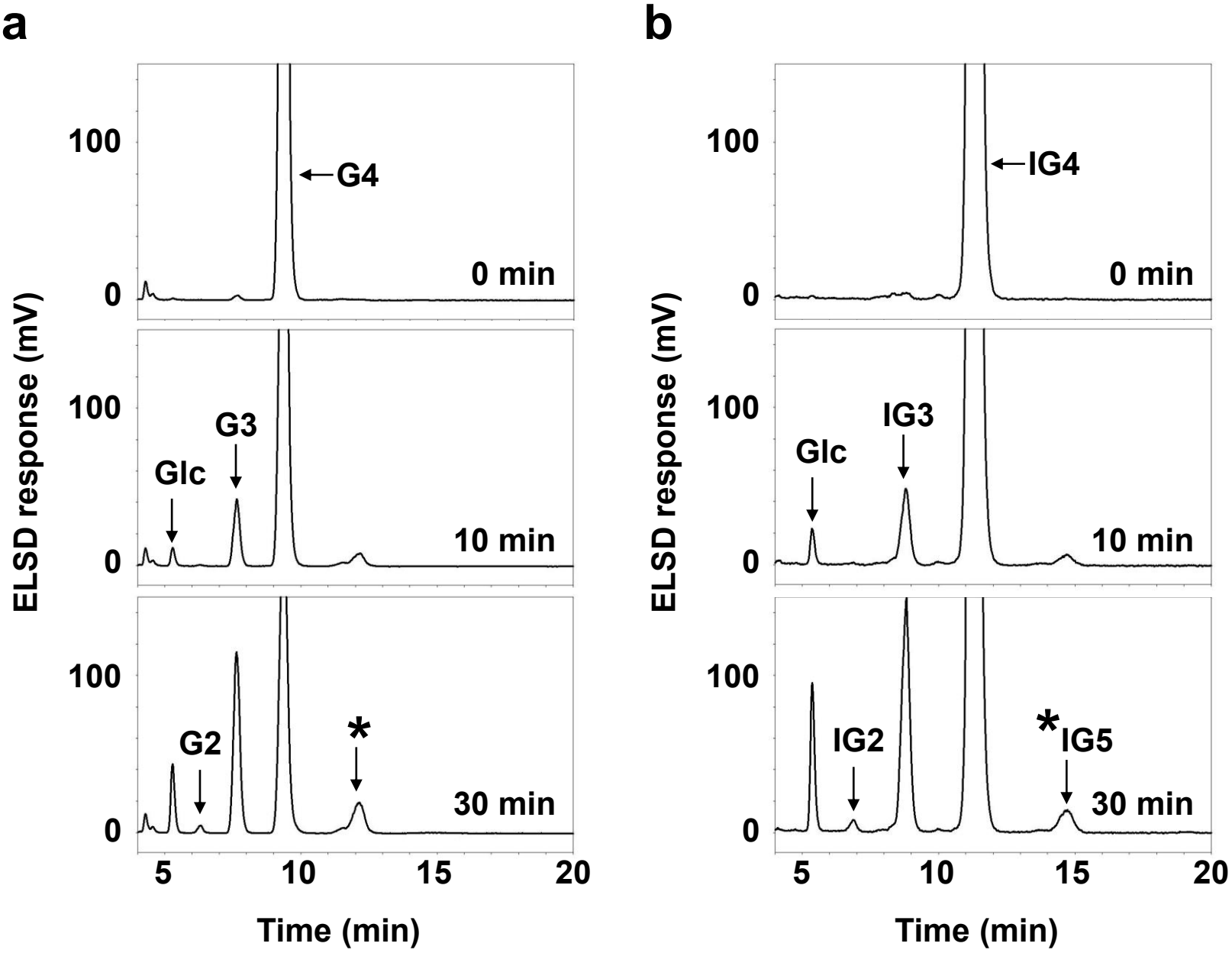
a
b
ELSD response (mV)
100
0
G4
0 min
Glc
G3
10 min
G2
*
30 min
IG4
IG3
IG2
*IG5
5
10
15
20
Time (min)

Fig. 6

Figure

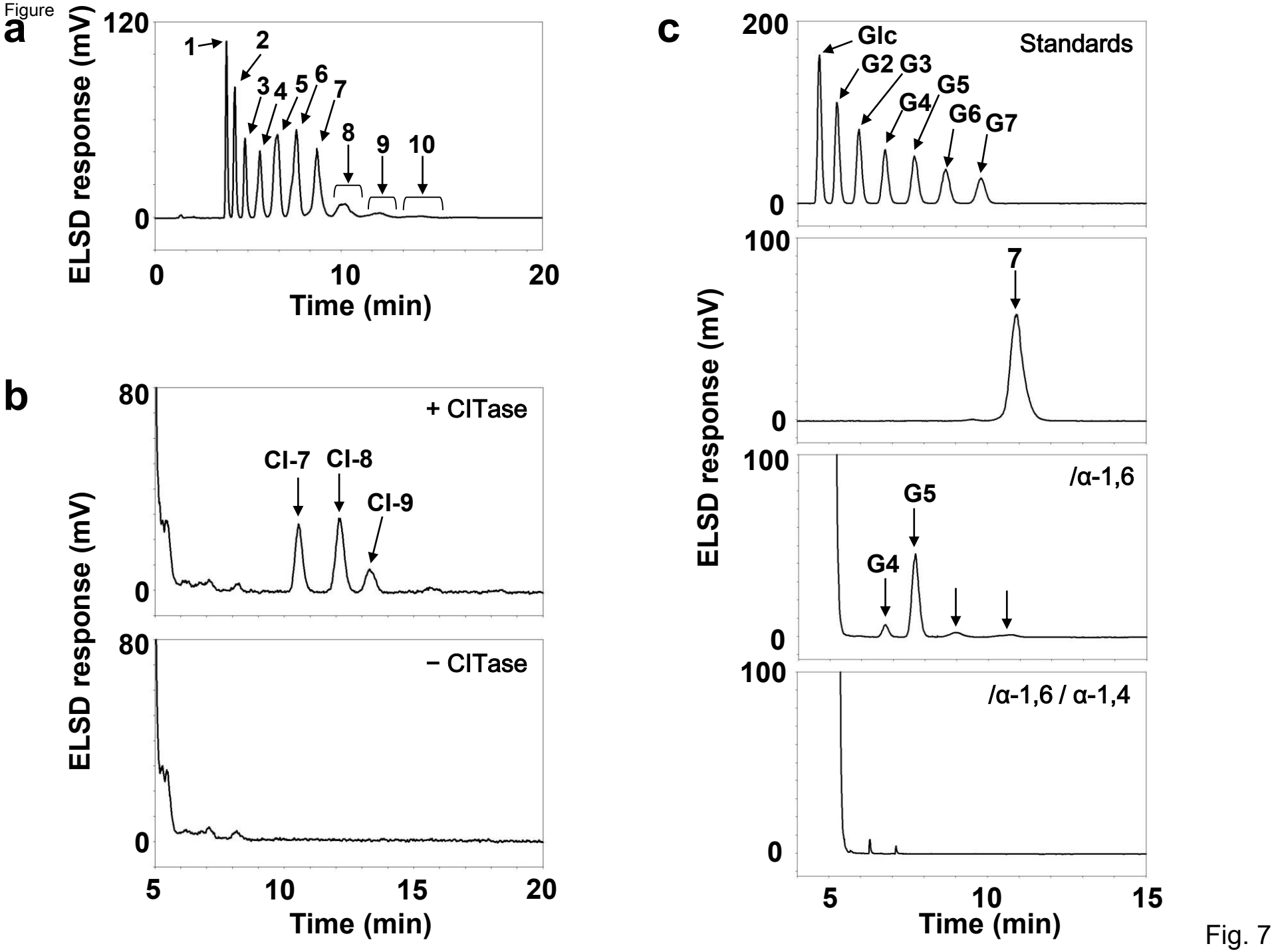
a
120
0
ELSD response (mV)
1
2
3
4
5
6
7
8
9
10
0
10
20
Time (min)
b
80
0
+ CITase
CI-7
CI-8
CI-9
80
0
− CITase
ELSD response (mV)
5
10
15
20
Time (min)
c
200
0
Glc
G2
G3
G4
G5
G6
G7
Standards
100
0
7
100
0
/α-1,6
G4
G5
100
0
/α-1,6 / α-1,4
ELSD response (mV)
5
10
15
Time (min)

Fig. 7