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Extracellular production of cycloisomaltooligosaccharide glucanotransferase and cyclodextran by a protease-deficient *Bacillus subtilis* host–vector system

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Abstract A cycloisomaltooligosaccharide (CI; cyclodextran) production system was developed using a *Bacillus subtilis* expression system for the cycloisomaltooligosaccharide glucanotransferase (CITase) gene. The CITase gene of *Bacillus circulans* T-3040, along with the α-amylase promoter (*PamyQ*) and *amyQ* signal sequence of *Bacillus amyloliquefaciens*, was cloned into the *Bacillus* expression vector pUB110 and subsequently expressed in *B. subtilis* strain 168 and its alkaline (*aprE*) and neutral (*nprE*) protease-deficient strains. The recombinant CITase produced by the protease-deficient strains reached 1 U/mL in the culture supernatant within 48 h of cultivation, which was approximately 7.5 times more than that produced by the industrial CITase-producing strain *B. circulans* G22-10 derived from *B. circulans* T-3040. When *aprE*- and *nprE*-deficient *B. subtilis* 168 harboring the CITase gene was cultured with 10% dextran 40 for 48 h, 17% of the dextran in the culture was converted to CIs (CI-7 to CI-12), which was approximately three times more than that converted by *B. circulans* G22-10 under the same dextran concentration. The *B. subtilis* host–vector system enabled us to produce CIs by direct fermentation of dextran along with high CITase production, which was not possible in *B. circulans* G22-10 due to growth inhibition by dextran at high concentrations and limited production of CITase.

Keywords Cyclodextran · Cycloisomaltooligosaccharide · Cycloisomaltooligosaccharide glucanotransferase · *Bacillus circulans* · *Bacillus subtilis*

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Introduction

Cyclodextrans are cycloisomaltooligosaccharides (CIs) consisting of 7 to 17 molecules of α-1,6-linked glucose and are represented as CI-7 to CI-17 (Oguma et al. 1993; Funane et al. 2007a, 2008). CIs strongly inhibit glucansucrase activity in mutans streptococci (Kobayashi et al. 1995), suggesting that CIs are antiplaque materials that would be useful in preventing dental caries. Among CIs, cycloisomaltodecaose (CI-10) shows an inclusion ability on par with cyclodextrins, as determined by its ability to stabilize the color of Victoria blue B (Funane et al. 2007a).

CIs are produced from dextran, and this reaction is catalyzed by cycloisomaltooligosaccharide glucanotransferase (CITase; EC 2.4.1.248) (Oguma et al. 1994). *Bacillus circulans* T-3040 [FERM BP-4132 (NBRC)] simultaneously secretes CITase and CIs into its culture supernatant when grown with dextran (Oguma et al. 1994). However, CITase production by *B. circulans* strains is very low (0.001 U/mL) and not sufficient for commercial production of CIs. To improve productivity, *B. circulans* T-3040 was mutagenized. *B. circulans* strain G22-10, which was obtained by treatment with nitrosoguanidine and streptomycin, has approximately 110 times more CITase activity than the parental strain (Kawabata et al. 2006; Funane et al. 2007b). Thus, *B.*

circulans G22-10 can be used for the efficient commercial production of CIs. However, dextran, the substrate for CI production, inhibits the growth of *B. circulans* T-3040 and its mutants. *B. circulans* G22-10 requires a long culture period of at least 4 days to produce sufficiently large amounts of CIs for practical use. Immobilization of *Escherichia coli* recombinant CITase facilitates CI production, resulting in sufficient yields in 10–16 h (Kawamoto et al. 2001). However, production of CIs by simple fermentation without much equipment and technology is desired for practical use.

Bacillus subtilis has been successfully used as a host for high-level expression of genes for various enzymes (Westers et al. 2004; Inoue et al. 2002; Yamamoto et al. 2005; Fahnestock 1986). Extracellular production of CITase using *B. subtilis* is a potential strategy to address the problems associated with *B. circulans* and immobilized CITase and produce CIs by fermentation. In the present study, CITase was produced and secreted by *B. subtilis* strains carrying the CITase gene, which was under the control of the *Bacillus amyloliquefaciens* α-amylase promoter (*PamyQ*). This system successfully produced 7.5 times more extracellular CITase than *B. circulans* G22-10. Moreover, CIs were simultaneously produced by direct fermentation of dextran at 2%, 5%, and 10% concentrations using this system.

Materials and methods

Strains

B. subtilis Marburg 168 (*trpC2*) and *B. amyloliquefaciens* NBRC 15535 were used in this study. Alkaline (*aprE*) and neutral (*nprE*) protease-deficient strains were produced by transforming wild-type *B. subtilis* 168 cells with genomic DNA of *B. subtilis* Marburg 168 (*trpC2*) strains in which *aprE* and *nprE* were disrupted by Spc^r and Neo^r cassettes (Takano Foods Co., Ltd., Omitama, Japan). Transformants were selected on Luria Bertani (LB) agar (Becton, Dickinson and Company, Sparks, MD, USA) plates supplemented with antibiotics (100 μg/mL spectinomycin and 7.5 μg/mL neomycin). Protease activities were assayed as described previously (Amory et al. 1987) with a minor modification using the chromogenic substrate azocasein (Sigma-Aldrich, Saint Louis, MO, USA). Culture supernatants were incubated in 80 mM Tris–HCl (pH 8.0) and 0.4% azocasein at 37°C for 30 min. The reaction was terminated by the addition of 6% trichloroacetic acid. After centrifugation at 10,000×*g* for 10 min, the supernatants were neutralized with NaOH, and the optical densities (ODs) of the samples were measured at 440 nm. One unit of the enzyme hydrolyzes 1 μg of azocasein per minute.

Construction of CITase gene-carrying vector and transformation of *B. subtilis* strains

To achieve extracellular expression in *B. subtilis*, the CITase gene of *B. circulans* T-3040 (GenBank ID: D61382) was fused with the regulatory region of *B. amyloliquefaciens amyQ* and the *amyQ* signal sequence (GenBank ID: V00092). The CITase expression vector pQS-CIT was constructed as follows: first, *PamyQ* and the *amyQ* signal peptide region were amplified using primers 1 [5′-AAAGGATCCGCCCCGCACATACGAAAAGACTGG-3′ (*Bam*HI site is underlined)] and 2 [5′- CGATGCCGCCA GAGCCTGAGGCTGATGTTTTTGTAATCG −3′] with chromosomal DNA from *B. amyloliquefaciens* as the template. Second, the CITase gene without the signal peptide sequence was amplified using primers 3 [5′-TCAGGCTCTGGCGGCATCGAGC-3′] and 4 [5′-AAATCTAGACTAGCTCACATTGATCCCGAAG −3′ (*Xba*I site is underlined)] with chromosomal DNA from *B. circulans* T-3040 as the template. Overlap polymerase chain reaction (PCR) was performed using primers 1 and 4 with a mixture of the amplified fragments as a template. The resulting PCR fragment was digested with *Bam*HI and *Xba*I and ligated into pUB110 at the same restriction sites using the linear form ligation method (Tsuge et al. 2003). The final CITase expression vector pQS-CIT is shown in Fig. 1.

B. subtilis strains were transformed with pQS-CIT by the method of Anagnostopoulos and Spizizen (1961). Kanamycin- and phleomycin-resistant transformants were screened on blue dextran-containing LB plates [1% peptone, 0.5% yeast extract, 1% NaCl, 0.8% dextran 40 (GE Healthcare, Uppsala, Sweden), 0.2% blue dextran 2000 (GE Healthcare), and 1.5% agar] supplemented with 10 μg/mL of kanamycin and 1 μg/mL of phleomycin. Each CITase-producing clone made a halo around the colony, which was formed by blue dextran 2000 degradation. DNA sequences of the inserted genes in the selected clones were confirmed using an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA, USA) and an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems).

Cultivation of pQS-CIT-carrying *B. subtilis* strains and preparation of extracellular and intracellular fractions

B. subtilis strains harboring pQS-CIT were precultured using a Bio Shaker BR 40 L (Taitec Co. Ltd., Koshigaya, Japan) with shaking at 200 rpm at 37°C for 24 h in 2 mL of mineral salts-supplemented P4M2 medium [4% peptone, 2% maltose, and 1% yeast extract containing 0.001% of NaCl, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, and 0.005% $CaCl_2 \cdot 2H_2O$ (pH 7.2)] with 10 μg/mL of kanamycin and 1 μg/mL of phleomycin. The cells were grown in 14-mL

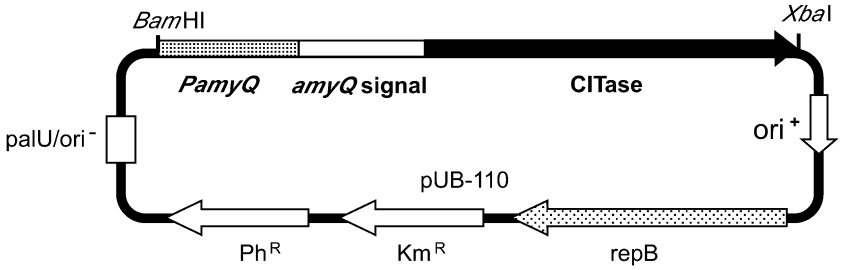

Fig. 1 Structure of the CITase expression vector pQS-CIT. pQS-CIT was constructed using pUB110. *PamyQ* is the promoter region of the α-amylase gene of *B. amyloliquefaciens*; the *amyQ* signal sequence is the signal peptide region of the α-amylase gene of *B. amyloliquefaciens*; *CITase* is the CITase gene of *B. circulans* T-3040 without its signal sequence; ori^+ and $palU/ori^-$ are replication origins; Km^R is the kanamycin resistance gene; Ph^R is the phleomycin resistance gene; *repB* is the replication initiator protein B; and *pUB110* is pUB110 digested with *Bam*HI and *Xba*I

tubes until late log phase. Each preculture was inoculated into 5.5 mL of the same medium containing 1 μg/mL phleomycin in L-shaped tubes. The initial absorbance at 600 nm was adjusted to an OD of 0.04. The cells were cultured with a TN-1506 biophotorecorder (Advantec MFS, Inc., Dublin, CA, USA) with shaking at 70 rpm at 37°C. The culture was centrifuged at 10,000×*g* for 15 min, and the supernatants were pooled as an extracellular fraction. Precipitated cells were washed two times with 20 mM Tris–HCl buffer (pH 7.0), suspended in the same buffer containing lysozyme (10 mg/mL), and incubated at 37°C for 30 min with gentle mixing. Subsequently, 100 μL of BugBuster (Merck KGaA, Darmstadt, Germany) and 1 μL of DNase I (Takara Bio Inc., Otsu, Japan) were added to the cell suspension and mixed. The cell lysate was centrifuged at 10,000×*g* for 15 min to remove cell debris and pooled as an intracellular fraction.

Cultivation of the *B. circulans* G22-10 strain and preparation of the extracellular fraction

B. circulans G22-10 was precultured with shaking at 200 rpm at 30°C for 2 days in 2 mL of LB broth (Becton, Dickinson and Company) containing 2% dextran 40 as described above. The preculture was inoculated into 5.5 mL of the same medium in L-shaped tubes. The initial absorbance at 600 nm was adjusted to an OD of 0.04 and cultured with shaking at 70 rpm at 30°C with the TN-1506 biophotorecorder. The culture was centrifuged at 10,000×*g* for 10 min, the precipitants were discarded, and the supernatants were pooled as an extracellular fraction.

Purification of CITase

B. subtilis 168 transformed with pQS-CIT and *B. circulans* G22-10 were cultured in 1 L mineral salts-supplemented P4M2 medium and 1 L LB broth containing 2% dextran 40, respectively, as described in the "Materials and methods" section, in 5 L baffled-bottom Erlenmeyer flasks with a shaker (TB-128R; Takasaki Scientific Instruments Corp., Kawaguchi, Japan) at 160 rpm. The growing conditions were 37°C for 2 days for *B. subtilis* and 30°C for 3 days for *B. circulans*. Culture supernatants were collected by centrifugation (5,000×*g* for 10 min), brought to 20–80% saturation with ammonium sulfate, and centrifuged at 10,000×*g* for 20 min. The resulting pellets were suspended in an appropriate volume of 50 mM sodium phosphate buffer (pH 8.0) and dialyzed against the same buffer. The supernatants were collected by centrifugation (10,000×*g* for 20 min) and loaded onto a Resource Q column (6 mL; GE Healthcare) equilibrated with 50 mM sodium phosphate buffer (pH 8.0). The column was washed with the same buffer, and the enzyme was eluted with a 0–0.6 M linear NaCl gradient. The eluted fraction was loaded onto a gel filtration column (Superose 12, 1.0×30 cm; GE Healthcare) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. Purified enzyme produced by *B. circulans* G22-10 strain was obtained at this step. For the enzyme produced by pQS-CIT-carrying *B. subtilis* 168, four times the fraction volume of 50 mM sodium phosphate buffer (pH 8.0) was added to the eluted fraction from the Superose 12 column. It was loaded onto a Mono Q column (1 mL; GE Healthcare) equilibrated with 50 mM sodium phosphate buffer (pH 8.0), washed with the same buffer, and the enzyme was eluted with a 0–0.6 M linear NaCl gradient to obtain purified enzyme. Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin used as a standard.

Assay of CITase activity and CI production

CITase activity was determined by measuring the amounts of CI-7, CI-8, and CI-9 produced from dextran as described previously (Yamamoto et al. 2006). The CITase assays were performed in 40 mM sodium acetate buffer (pH 5.5) containing 10 mM $CaCl_2$ and 2% dextran 40 at 40°C. CIs in the reaction mixtures were measured by high-

performance liquid chromatography (HPLC; CLASS-VP with Evaporative Light Scattering Detector System; Shimadzu, Kyoto, Japan) using a TSK gel Amide-80 column (4.6×250 mm; Tosoh, Tokyo, Japan). The mobile phase was acetonitrile–water (55:45, *v*/*v*), and the flow rate was 1 ml/min. One unit of CITase activity was defined as the amount of enzyme that produced 1 μmol of the sum of CI-7, CI-8, and CI-9 per minute.

The amount of CI (CI-7 to CI-12) produced in the culture supernatants was measured using HPLC as described above. The standard CIs were purchased from C-I Bio, Ltd. (Tomigusuku, Okinawa, Japan).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and zymography

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using an 8% polyacrylamide gel following the method of Laemmli (1970). Protein bands were stained with Coomassie Brilliant Blue R-250 (CBB). Enzyme zymography was performed according to the method of Igarashi et al. (1992) with a slight modification. Proteins were separated on an 8% SDS-PAGE gel containing 0.5% blue dextran 2000. Subsequently, the proteins in the gels were renatured by washing the gel two times with water and then soaking it in 50 mM sodium acetate buffer (pH 5.5) at 37°C until active CITase was detected by the formation of a clear band on a blue background.

Results

Expression of the CITase gene from *B. circulans* T-3040 in the *B. subtilis* 168 strain

To express the CITase gene from *B. circulans* T-3040 in *B. subtilis* and produce extracellular CITase, an expression vector (pQS-CIT) was constructed containing *PamyQ* and the *amyQ* signal sequence of *B. amyloliquefaciens* situated upstream of the CITase gene (Fig. 1). The *B. subtilis* Marburg 168 strain carrying pQS-CIT mostly produced CITase in the extracellular fraction after 48 h of cultivation, with only a trace amount of CITase activity detected in the intracellular fraction. The *B. subtilis* 168 strain without pQS-CIT and the strain harboring only pUB110 showed no CITase activity in either the extracellular or intracellular fraction.

The 168 strain grew to an OD of approximately 30 at an absorbance of 600 nm (Fig. 2a) and exhibited CITase activity of 0.682±0.057 U/mL in the culture medium after 36 h of cultivation (Fig. 2b). This value was 15 times higher than that of *B. circulans* G22-10 after 36 h of cultivation (0.0408±0.017 U/mL) and approximately 5 times higher than that of the same strain after 48 h of cultivation (0.139±0.045 U/mL). Purified CITase produced by the G22-10 strain and *B. subtilis* recombinant CITase possessed almost the same specific activities (Table 1). Higher CITase activity in the pQS-CIT-carrying *B. subtilis* 168 culture supernatants could be due to the production of large amounts of enzyme protein. However, the CITase activity of the 168 strain decreased after 48 h of cultivation.

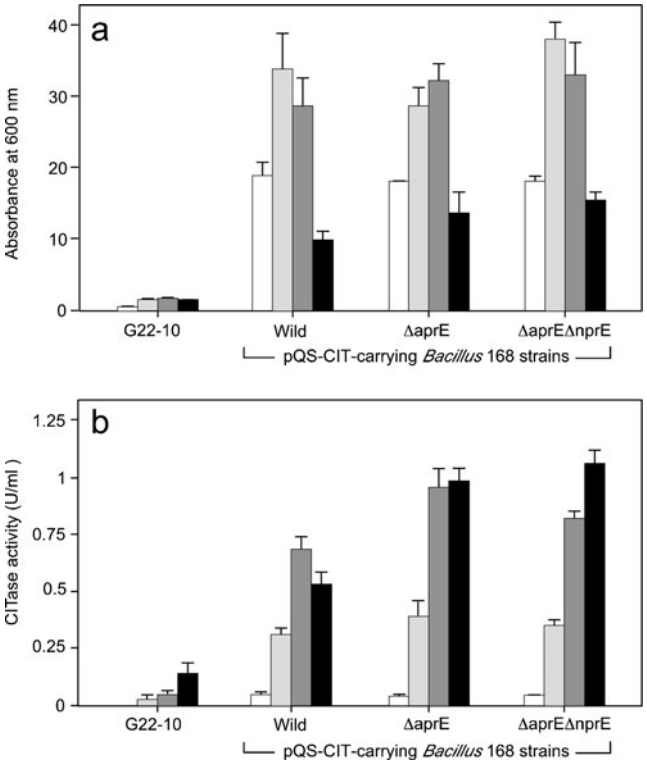

Fig. 2 Growth (**a**) and CITase production (**b**) by the *B. circulans* G22-10 and pQS-CIT-carrying *B. subtilis* strains. Growth was assessed as culture turbidity by measuring absorbance at 600 nm. CITase activity was determined as described in the "Materials and methods" section. *White bar*, 12-h culture; *light gray bar*, 24-h culture; *dark gray bar*, 36-h culture; and *black bar*, 48-h culture. The *error bars* indicate the standard deviation of triplicate experiments. *G22-10* is *B. circulans* G-22-10; *Wild* is *B. subtilis* 168; *ΔaprE* is *B. subtilis* 168 *ΔaprE*; and *ΔaprEΔnprE* is *B. subtilis* 168 *ΔaprEΔnprE*

Expression of the CITase gene in protease-deficient *B. subtilis* 168 strains

We hypothesized that the reduction in CITase activity in the later stage of cultivation was caused by proteolysis by the host proteases secreted into the medium. Kawamura and Doi (1984) reported that extracellular protease activity was reduced by 65% in the *aprE*-deficient (*ΔaprE*) mutant, and the *aprE* and *nprE* double-deficient (*ΔaprEΔnprE*) mutant exhibited 96% decrease. Thus, to prevent CITase degradation, *aprE*- and/or *nprE*-deficient mutants were used as host

Table 1 Purification of CITase from culture supernatants of *B. circulans* G22-10 (a) and pQS-CIT-carrying *B. subtilis* 168 Δ*aprE*Δ*nprE* (b)

	Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
a	Culture sup.[a]	1.08×10^{4}	71.8	0.007	100.0	1
	20–80% $(NH_4)_2SO_4$ ppt.	1.66×10^{3}	20.9	0.013	29.1	2
	Resource Q	1.96	6.73	3.43	9.4	490
	Superose 12	1.00×10^{-1}	1.42	14.2	2.0	2,029
b	Culture sup.[a]	4.03×10^{4}	541	0.013	100.0	1
	20–80% $(NH_4)_2SO_4$ ppt.	9.70×10^{3}	335	0.345	61.9	27
	Resource Q	5.65×10	315	5.58	58.2	429
	Superose 12	2.70×10	169	6.26	31.2	482
	Mono Q	4.54	66.5	14.6	12.3	1,123

[a] Culture supernatant from 1 L culture

strains. In this study, the Δ*aprE* and Δ*aprE*Δ*nprE* mutants obtained from the *B. subtilis* 168 strain also exhibited protease activities reduced by approximately 65% and 90%, respectively. Furthermore, the CITase activities of Δ*aprE* and Δ*aprE*Δ*nprE* mutants reached 0.980±0.070 U/mL and 1.062±0.059 U/mL, respectively, approximately two times higher than the parental strain, and the activities did not decrease until after 48 h of cultivation (Fig. 2b).

Zymography analysis showed that the protein bands of the protease-deficient mutants were thicker than those of the parental strain (Fig. 3a). The molecular size of CITase is predicted to be 103 kDa, which is in agreement with that of the protein band in the CBB-stained gel (Fig. 3a) and the halos observed by zymography (Fig. 3b). Two smaller bands with a halo at 96 and 88 kDa were also observed by zymography.

The CITase activity of the *B. subtilis* Δ*aprE*Δ*nprE* culture supernatant was 7.5 times greater than that of the *B. circulans* G22-10 culture supernatant, and the specific activity of the *B. subtilis* Δ*aprE*Δ*nprE* culture supernatant was almost twice that of the *B. circulans* G22-10 culture supernatant (Table 1). Both CITases were purified as 103 kDa proteins, and their specific activities were approximately equal, with 14.6 U/mL for *B. subtilis* Δ*aprE*Δ*nprE* recombinant CITase and 14.2 U/mL for *B. circulans* G22-10 CITase (Table 1).

Production of CIs using the pQS-CIT-carrying protease-deficient Δ*aprE*Δ*nprE* mutant

CI (CI-7 to CI-12) production was measured in the culture supernatant of pQS-CIT-carrying *B. subtilis* 168 Δ*aprE*Δ*nprE* grown in mineral salts-supplemented P4M2 medium with 2%, 5%, and 10% dextran 40. CI production was compared with that of *B. circulans* G22-10 strains grown in 2%, 5%, and 10% dextran 40-supplemented LB broth. As shown in Fig. 4a, dextran 40 inhibited the growth of the G22-10 strain, and thus, its CI production level was not enhanced by an increasing dextran 40 concentration (Fig. 4b). The G22-10 strain grown with 2%, 5%, and 10% dextran 40 for 48 h produced CIs (sum of CI-7 to CI-12) at yields of 6.02±0.69 mg/mL, 5.30± 0.41 mg/mL, and 5.43±0.27 mg/mL, respectively.

In contrast, the growth of the Δ*aprE*Δ*nprE* mutant was apparently unaffected by dextran 40, and no reduction in cell density was observed after 48 h of growth with 10% dextran in the medium (Fig. 4a). In addition, CI production by the Δ*aprE*Δ*nprE* mutant was enhanced with increasing dextran 40 concentration (Fig. 4b). CIs (CI-7 to CI-12) were produced after 48 h of cultivation from 2%, 5%, and 10% dextran 40 in the amounts of 8.59±0.32 mg/mL, 12.27±0.22 mg/mL, and 16.62± 1.14 mg/mL, respectively. The yields of CIs from the conversion of the substrate dextran were 43%, 25%, and 17%, respectively.

Discussion

Using *PamyQ* and the *amyQ* signal sequence, the CITase gene was successfully expressed extracellularly by *B. subtilis*. In previous studies, the CITase gene has been expressed in *E. coli* BL21 (DE3) using pET systems (Yamamoto et al. 2006; Funane et al. 2011). However, there are disadvantages in the use of *E. coli* expression systems for CITase and CI production. For example, a low temperature must be maintained during expression; 18°C in Yamamoto et al. (2006) or 25°C in Funane et al. (2011). At higher temperatures, the CITase gene is barely expressed in *E. coli* and the recombinant CITase becomes inactive inclusion bodies. Another problem is that the enzyme accumulates inside the *E. coli* cells, and the cells must be disrupted to use the enzyme. To produce CIs efficiently using *E. coli* recombinant CITase, the enzyme was immobilized on Chitopearl BCW-3505 (Kawamoto et al. 2001), resulting in an enzyme activity of 1.75 U/ml carrier.

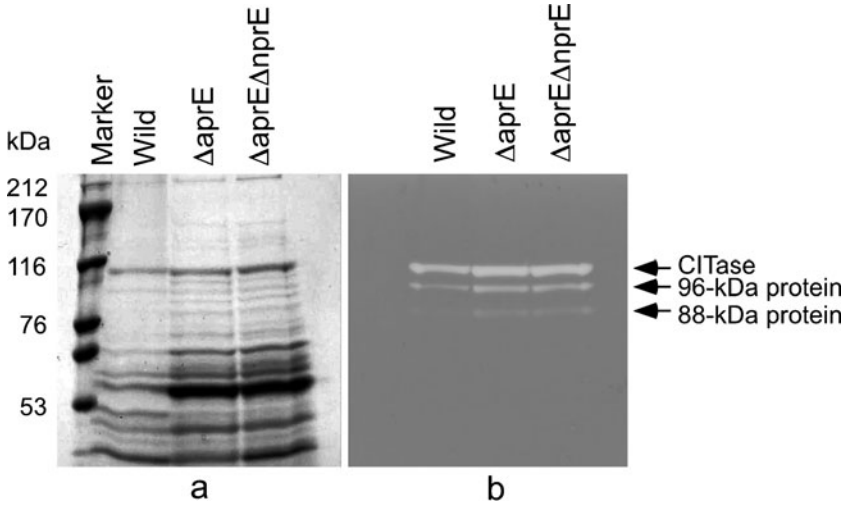

Fig. 3 SDS-PAGE (**a**) and zymography (**b**) of the culture supernatant of pQS-CIT-carrying *B. subtilis* 168 and its protease-deficient strains. The *B. subtilis* transformants were grown in mineral salts-supplemented P4M2 medium at 37°C for 48 h. Each culture supernatant (10 μL) was loaded onto an 8% (*w*/*v*) polyacrylamide gel with or without 0.5% blue dextran 2000. After electrophoresis, the gel without blue dextran was stained with CBB. The blue gel was washed two times with water with gentle shaking for 30 min and subsequently incubated in 50 mM sodium acetate (pH 5.5) at 37°C for 3 h. Lanes *Marker*, *Wild*, *ΔaprE*, and *ΔaprEΔnprE*: Amersham high molecular weight calibration kit for SDS electrophoresis (GE Healthcare), *B. subtilis* 168, *B. subtilis* 168 Δ*aprE*, and *B. subtilis* 168 Δ*aprE*Δ*nprE*, respectively

They calculated the yield of CIs as the sum of CI-7, CI-8, and CI-9 produced versus the amount of substrate dextran and found that the maximum conversion yield of CIs (CI-7 to CI-9) in batch reactions for 2% and 10% dextran was 24% and 12%, respectively. We have shown that a pQS-CIT-carrying *B. subtilis* 168 Δ*aprE*Δ*nprE* strain yields CIs (CI-7 to CI-9) from 2% and 10% dextran at 24% and 11%, respectively, after 48 h of cultivation. The CI production

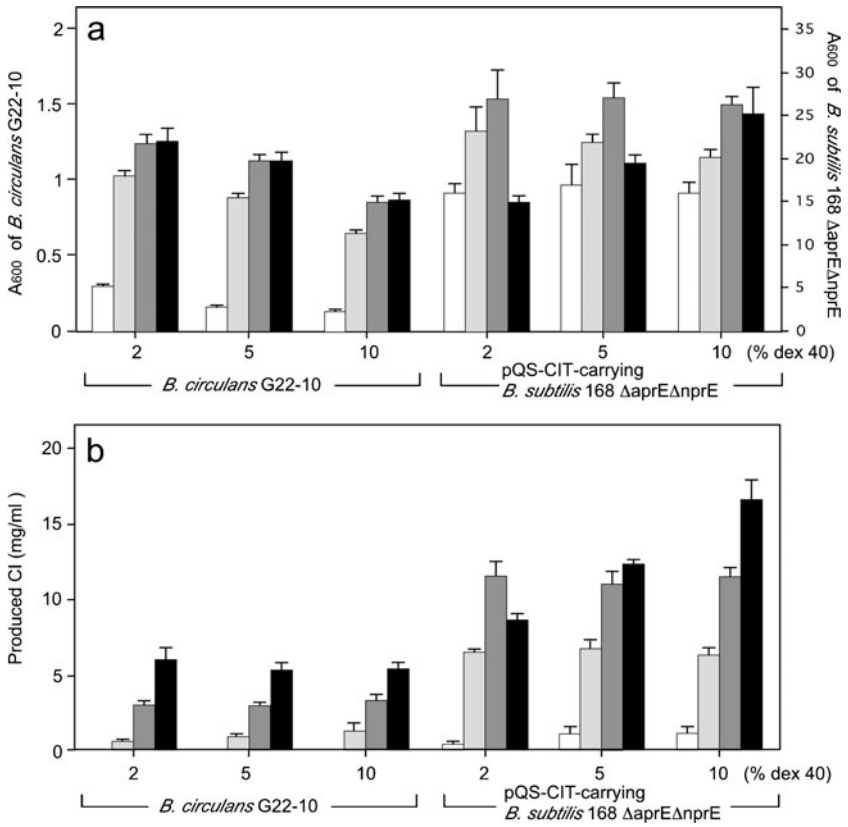

Fig. 4 Growth (**a**) and CI production (**b**) by *B. circulans* G22-10 and pQS-CIT-carrying *B. subtilis* 168 Δ*aprE*Δ*nprE* strains. Growth was measured as described in the legend of Fig. 2. The CI produced (mg/mL) is indicated as the sum of CI-7 to CI-12 per milliliter of culture broth. *White bar*, 12-h culture; *light gray bar*, 24-h culture; *dark gray bar*, 36-h culture; and *black bar*, 48-h culture. The *error bars* indicate the standard deviation of triplicate experiments. *% dex 40* is dextran 40 concentration (percent) in culture broth

level of this strain is nearly identical to that of the immobilized CITase system.

B. circulans T-3040 and U-155 are known as CI- and CITase-producing strains (Oguma et al. 1994; Oguma and Kawamoto 2003). The optimum growth temperature of these strains is 30°C, and they do not grow well at higher temperatures. CITase activity in *B. circulans* T-3040 is only 0.001 U/mL, whereas the high CITase-producing mutant *B. circulans* G22-10 has 110 times more activity. However, it is still low at 0.110 U/mL after 3 days of culturing (Kawabata et al. 2006; Funane et al. 2007b). CITase is an inducible enzyme, and dextran is required for enzyme induction in *B. circulans* strains. However, dextran inhibits cell growth at high concentrations (Fig. 4).

Our study revealed that the *B. subtilis* 168 Δ*aprE*Δ*nprE* mutant strain produces 1.062±0.0590 U/mL CITase in a 48-h culture (Fig. 2b), and its specific activity is as strong as *B. circulans* G22-10 CITase (Table 1). CITase activity in the culture supernatant was not decreased using the protease-deficient strains as host cells (Figs. 2 and 3), which suggests that the decrease in CITase activity in the protease-positive host cells was due to proteolysis by AprE and NprE. The 96- and 88-kDa protein bands in the zymogram were observed in the samples from both the protease-positive- and -negative hosts. A previous deletion study of T-3040 CITase revealed that when the enzyme lacks the 234-amino acid C-terminal variable region (78 kDa), it retains its CI-producing activity with the same k_{cat} values as the wild-type enzyme, but further C-terminal deletion completely abolishes the enzyme activity (Funane et al. 2011). The 96- and 88-kDa proteins in the zymogram were larger than the 78-kDa deletion mutant protein. These proteins may be CITases with truncated C-terminal regions that were partially digested by remnant proteases secreted by the host cells. When the wild-type *B. subtilis* 168 strain was used as a host, its proteases, including AprE and NprE, probably digested CITase until it was no longer active, causing the decrease in CITase activity after 48 h of cultivation (Fig. 2b).

Currently, CIs are commercially produced from dextran using CITase obtained by culturing *B. circulans* G22-10. However, production of sufficiently large amounts of CITase from the G22-10 strain for practical use is time consuming. The growth of the CI-producing *B. circulans* is inhibited by the substrate dextran, limiting CI production by direct CI fermentation with *B. circulans* (Fig. 4). In this study, we demonstrated a simple method for producing CI by cultivating a strain of *B. subtilis* with dextran. As dextran concentration increased, increased CI production was observed with this system. Notably, since CITase produces larger molecules of CIs up to CI-17 (Funane et al. 2008), the conversion efficiency to CIs may be larger than the yield calculated from the production of CI-7 to CI-12. Based on our findings, this system could be used for the industrial production of CIs along with CITase by direct fermentation.

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