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Cloning and heterologous expression of the *ftf*CNC-2(1) gene from *Weissella confusa* MBFCNC-2(1) as an extracellular active fructansucrase in *Bacillus subtilis*

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Fructan-exopolysaccharides (fructan-EPS) (inulin and levan) and their oligosaccharides (fructooligosaccharides, FOS) have drawn considerable interest in the food and pharmaceutical industries. EPS-producing lactic acid bacteria have been reported to produce β-fructans (inulin and levan), as well as α-glucans, by the function of sucrase enzymes, i.e., fructansucrase and glucansucrase. A fructansucrase *ftf*CNC-2(1) gene from *Weissella confusa* strain MBFCNC-2(1) was previously cloned in *Escherichia coli*. In this study, we aimed to express the *ftf*[CNC-2(1)] gene in *Bacillus subtilis* to obtain the active form of the extracellular recombinant protein FTF[CNC-2(1)]. This cloning was achieved by inserting the gene in-fusion with the signal sequence of the *B. subtilis* subtilisin E. SDS-polyacrylamide gel electrophoresis analysis and *in situ* activity assay with Periodic Acid-Schiff staining revealed that the recombinant FTF[CNC-2(1)] was successfully expressed as an extracellular protein from *B. subtilis* DB403 in its active form, which was confirmed using sucrose and raffinose.

[**Key words:** Fructansucrase; Fructosyltransferase; *ftf*; Inulosucrase; *Weissella confusa*; *Bacillus subtilis*]

Fructan-exopolysaccharides (fructan-EPS) (inulin and levan) and their oligosaccharides (fructooligosaccharides, FOS) are well known for their uses in various industrial fields in the food and pharmaceutical industries. For example, inulin is widely used in the pharmaceutical industry as a protein protectant (1,2). Another type of EPS-fructan is levan. This biopolymer is known for its wide uses in industries such as medicine, cosmetics and food. Bacterial EPS-fructans have been produced as a biopolymer with a higher degree of polymerization (DP) compared to the vegetable-derived fructans; therefore, they will serve as an excellent alternative source to obtain inulin polymer.

Sucrase enzymes, i.e., fructansucrase (FS) and glucansucrase (GS) in EPS-producing lactic acid bacteria (LAB), have been reported to produce β-fructans (inulin and levan) and α-glucans, respectively. There are two types of fructans using FS produced by LAB: levans, which mainly consist of β-(2 → 6)-linked fructose residues and occasionally contain β-(2 → 1)-linked branches, and inulin-type fructans, which consist of β-(2 → 1)-linked fructose residues with β-(2 → 6)-linked branches (3).

The FS structure has been divided into four regions based on their deduced amino acid sequences as previously described (3): (i) a signal peptide, (ii) an N-terminal stretch that varies in length, (iii) a conserved catalytic core of approximately 500 amino acids that is shared between all family GH68 members (carbohydrate-active enzyme website: http://afmb.cnrs-mrs.fr/CAZY), and (iv) a C-terminal stretch of various lengths, in some cases with a cell wall binding domain (LPXTG).

Bacterial inulin production, which is exclusively produced by LAB, has been reported in some cariogenic *Streptococcus mutans* and *Streptococcus salivarius* strains (4–6), *Leuconostoc citreum* CW28 (7), and *Lactobacillus reuteri* 121 (8,9). Bacterial levan, which is widely produced in gram-positive and negative bacteria, is produced by *Leuconostoc mesenteroides* (10), *L. reuteri* 121 (11,12), *Lactobacillus sanfranciscensis* (13–15), and streptococci (16–18). *Lactobacillus frumenti*, *Lactobacillus pontis*, *Lactobacillus panis* and *Weissella confusa* were also shown to produce fructans (13–15,19), but their fructan binding types have not been well characterized. The molecular masses of the produced fructans were reported to vary from 2×10^4 to 50×10^6 Da. There are some reports that the molecular mass of the EPS fructan produced is dependent of growth and incubation conditions, e.g., the temperature, salinity, and sucrose concentration used (20–22).

A local strain of *W. confusa*, MBFCNC2-(1), isolated previously from a traditional beverage *cincau*, was studied for EPS-producing LAB, and it was cloned for its fructansucrase-encoded genes, *ftf*CNC-2(1) (23). From the deduced amino acid sequence, it was revealed that the gene possessed a unique feature of FS compared to the genes in the currently available databases; MBFCNC2-(1) was determined to be a hybrid of GS and FS, in which the N-terminal variable region showed a similar sequence as the N-terminus of *gtf*Kg15 (putative glucansucrase) (24), i.e., MLRNNYFGET KTHYKLYKCG KNWAVMGISL FSLGLGMLVT SQPVSA. The catalytic core of FTFCNC-2(1) was identified as belonging to glycoside hydrolase 68, with highly conserved motifs of 480 amino acid (aa), which

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apparently corresponded to the sizes observed in microbial FTFs. Nonetheless, at its C-terminus, the LPXTG motif was also reported to occur in slightly different motifs from LPKAG, as reported for *Lactobacillus johnsonii* NCC 533 inulosucrase InuJ and *Lactobacillus gasseri* DSM 20243 and 20077 (25,26). However, the expression of this FS gene did not exhibit an active form of FS (23). We have also cloned the truncated version of this FS coding gene, but its heterologous expression was failed either, in *Escherichia coli* BL21 Star or *Bacillus subtilis* 168 (unpublished data). We think that one of the possibilities is incorrect folding or secretion of the recombinant protein probably due to the overexpression in *E. coli* cells or the lack of suitable signal sequence for *B. subtilis* cells (data not shown).

B. subtilis, despite its reported potential as a sucrase (i.e., FS levansucrase) carrier (27), has also been intensively developed for advanced cloning systems such as *B. subtilis* DB403. Additional heterologous recombinant proteins have been cloned more frequently and expressed by employing this alternative prokaryote expression host. *B. subtilis* as a gram-positive bacterium is phylogenetically very distant from *E. coli*. A *B. subtilis* cloning system, similar to the gram-negative bacterium *E. coli*, could permit interesting insight into the diverse processes that convert genetic information to the corresponding phenotype under very different cellular environments.

To obtain the recombinant FTF[CNC-2(1)] in the culture supernatant, the truncated *ftf*[CNC-2(1)] gene was cloned and expressed in *B. subtilis* under the signal sequence of subtilisin gene, *aprE* (28) by in-fusion cloning. Hence, the study of the expression of this *Weissella* FS will provide important information regarding the unique FS gene sequence data obtained from previous studies; furthermore, this study will also provide information regarding the potential ability to produce inulin-like fructan using recombinant *Weissella* FS.

MATERIAL AND METHODS

Bacterial strains and medium *E. coli* BW25113 was used as the host for DNA cloning, and *B. subtilis* DB403 was used as the host for expression. *E. coli* and *B. subtilis* were grown aerobically at 37°C in Luria–Bertani, LB medium (USB Affymetrix, USA) or SOC medium (Invitrogen, USA) in a high speed rotary shaker for transformation. LB medium composition was as follows: casein peptone 50% w/v, yeast extract 25% w/v and sodium chloride 25% w/v, whereas SOC was as follows: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose. For selection of transformants, LB agar medium was used by adding 1.5% agar supplemented with the appropriate antibiotic (10 μg/ml or 20 μg/ml tetracycline or 50 μg/ml ampicillin). Strains containing recombinant plasmids were cultivated in LB medium supplemented with the appropriate antibiotic.

Cloning strategy of truncated version and primer design The region targeted for truncated cloning was selected from nt 140 to 2700 in the *ftf* gene after in-frame aa residues based on the following information: (i) *ftf*CNC-2(1) DNA sequence information available in the NCBI database obtained from a previous study (A.N. ADB27748), (ii) the truncated *ftf* cloning strategy as previously described (26) and (iii) the alignment result with several *ftf* genes using the Conserved Domain Architecture Retrieval Tool (cdart, http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps), as presented in Fig. 1. The catalytic domain of FS genes is located at various aa positions in the protein fragment, indicating that in addition to FS activity, the protein could also have another function related to its specific substrates in an attempt to generate diverse EPS structures. PCR cloning was designed with the in-fusion cloning strategy using a signal sequence (SS) of the subtilisin gene, as presented in Fig. 2. Primers for PCR truncated *ftf* were designed carefully using Clone Manager, and the first oligonucleotides were chosen. All primers used in this study are listed in Table 1.

DNA manipulation The genomic DNA of *W. confusa* MBFCNC-2(1) was extracted using phenol + chloroform + isoamyl alcohol (PCIA), and it was prepared as previously described (23). For isolation and purification of amplicon DNA from agarose gels, Qiaquick PCR Clean Up kit (Qiagen, USA) was used. The genomic DNA of *W. confusa* MBFCNC-2(1) was used for PCR cloning. pIOE (28) and pHY300PLK (29) plasmids were isolated using the Wizard mini-prep kit (Promega, USA).

PCR cloning and in-fusion recombination To obtain subtilisin signal sequence (subtilisin SS) DNA fragment, PCR (A) was run containing 1 μl 10× buffer, 1.6 mM $MgSO_4$, 2 mM of each deoxyribonucleotide triphosphate (dNTP), 0.20 μg plasmid DNA pIOE, and 2 pmol of each primer (primer-1 and primer-3) using 1 Unit per reaction of KOD plus DNA polymerase (Takara, Japan). The PCR conditions were as follows: 94°C for 2 min, followed by 5 cycles of 94°C for 15 s, 65°C for 30 s and 72°C for 1.5 min. The obtained PCR product was run on an agarose gel. A 0.2 kb DNA fragment harboring Subtilisin SS obtained from PCR was gel purified using the Qiaquick Gel Extraction Purification kit (Qiagen), followed by restriction enzyme double digestion using *Eco*RI and *Bam*HI.

Another PCR (B) was run to obtain the truncated fragment of the *ftf* gene. Genomic DNA of MBFCNC-2(1) was used as a template using primer-2 and primer-4. The reaction mixture consisted of 1 μl 10× buffer, 1.6 mM $MgSO_4$, 2 mM of each deoxyribonucleotide triphosphate (dNTP), 0.50 μg gDNA of MBFNC-2(1), and 2 pmol of each primer using 1 Unit per reaction of KOD plus DNA polymerase (Takara). The PCR condition was 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 65°C for 1 min and 72°C for 3 min, with an extension step at 72°C for 10 min. DNA fragments with sizes larger than 2 kb harboring truncated *ftf*CNC-2(1) gene obtained from PCR

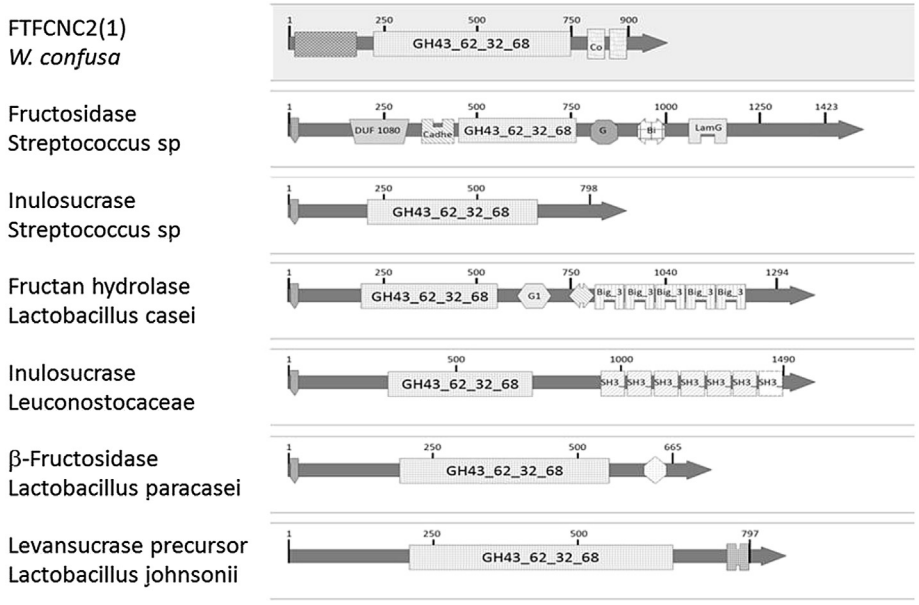

FIG. 1. Alignment of several genes coding for FS using Conserved Domain Architecture Retrieval Tool (cdart) results as a reference.