

Role of the *ganSPQAB* Operon in Degradation of Galactan by *Bacillus subtilis*

Hildegard Watzlawick, Kambiz Morabbi Heravi, Josef Altenbuchner

Institut für Industrielle Genetik, Universität Stuttgart, Stuttgart, Germany

ABSTRACT

Bacillus subtilis possesses different enzymes for the utilization of plant cell wall polysaccharides. This includes a gene cluster containing galactan degradation genes (*ganA* and *ganB*), two transporter component genes (*ganQ* and *ganP*), and the sugar-binding lipoprotein-encoding gene *ganS* (previously known as *cycB*). These genes form an operon that is regulated by GanR. The degradation of galactan by *B. subtilis* begins with the activity of extracellular GanB. GanB is an endo- β -1,4-galactanase and is a member of glycoside hydrolase (GH) family 53. This enzyme was active on high-molecular-weight arabinose-free galactan and mainly produced galactotetraose as well as galactotriose and galactobiose. These galacto-oligosaccharides may enter the cell via the GanQP transmembrane proteins of the galactan ABC transporter. The specificity of the galactan ABC transporter depends on the sugar-binding lipoprotein, GanS. Purified GanS was shown to bind galactotetraose and galactotriose using thermal shift assay. The energy for this transport is provided by MsmX, an ATP-binding protein. The transported galacto-oligosaccharides are further degraded by GanA. GanA is a β -galactosidase that belongs to GH family 42. The GanA enzyme was able to hydrolyze short-chain β -1,4-galacto-oligosaccharides as well as synthetic β -galactopyranosides into galactose. Thermal shift assay as well as electrophoretic mobility shift assay demonstrated that galactobiose is the inducer of the galactan operon regulated by GanR. DNase I footprinting revealed that the GanR protein binds to an operator overlapping the -35 box of the σ^A -type promoter of P_{gan} , which is located upstream of *ganS*.

IMPORTANCE

Bacillus subtilis is a Gram-positive soil bacterium that utilizes different types of carbohydrates, such as pectin, as carbon sources. So far, most of the pectin degradation systems and enzymes have been thoroughly studied in *B. subtilis*. Nevertheless, the *B. subtilis* utilization system of galactan, which is found as the side chain of the rhamnogalacturonan type I complex in pectin, has remained partially studied. Here, we investigated the galactan utilization system consisting of the *ganSPQAB* operon and its regulator *ganR*. This study improves our knowledge of the carbohydrate degradation systems of *B. subtilis*, especially the pectin degradation systems. Moreover, the galactan-degrading enzymes may be exploited for the production of galacto-oligosaccharides, which are used as prebiotic substances in the food industry.

Soil microorganisms are usually able to degrade a variety of polysaccharides and carbohydrates found in soil, including the remains of plants and especially the plant cell wall. The primary cell wall of plants is composed of different polysaccharide components, including cellulose, hemicellulose, and pectin, while the secondary cell walls are rigidified by lignin (1, 2). Among them, pectin is a family of galacturonic acid-rich polysaccharides that consists of the following three major pectic polysaccharide types: a linear component of polygalacturonan without branches called homogalacturonan (HG) and branched chains of rhamnogalacturonan type I (RG-I) and rhamnogalacturonan type II (RG-II) (Fig. 1). HG forms a linear backbone chain of α -1,4-linked D-galacturonic acid monomers, which are methyl esterified. Other pectic polysaccharides have the same HG backbone with D-xylose side residues in xylogalacturonan or with highly branched polysaccharides, including xylose, glucuronic acid, rhamnose, arabinose, apiose, galacturonic acid, fucose, and galactose, in RG-II. On the other hand, RG-I consists of a backbone chain of α -1,4-D-galacturonic acid- α -1,2-L-rhamnose with branches of arabinogalactan, galactan, and arabinan (3, 4) (Fig. 1).

Bacillus subtilis, as a common soil saprophytic bacterium, is able to degrade pectin and metabolize it through a RG-I degradation pathway (5). Principally, RG-I is degraded by the action of three types of enzymes, RG-I hydrolases, RG-I lyases, and RG-

I-specific acetyl esterases (for a review, see reference 6). To break down the backbone chain of the pectin polymer, *B. subtilis* encodes two pectate lyases, Pel (7) and PelC (8). Also, a putative pectate lyase (*ywoF*) and a pectin lyase (*pelB*) exist in the genome of *B. subtilis* (9, 10). At least two gene clusters, namely, *yesOPQRSTUVWXYZ* and *ytePRSTU*, are involved in the degradation of RG-I (5). Based on similar models proposed by Itoh et al. (11) and Silva et al. (6), the RG-I backbone is primarily deacetylated by the RG acetyl esterases YesT and YesY (12) and then attacked by extracellular RG endolyase YesW, cleaving the α -(1 \rightarrow 4) bonds of the RG-I backbone to produce unsaturated galacturo-

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Address correspondence to Kambiz Morabbi Heravi, kambiz.morabbi@iig.uni-stuttgart.de.

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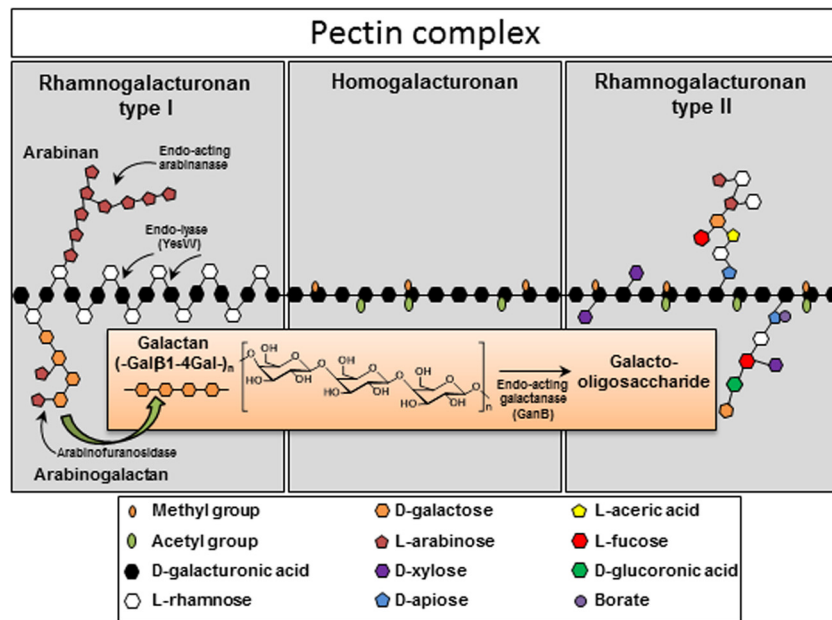


FIG 1 Structure of the pectin complex and degradation of the arabinogalactan side chains of rhamnogalacturonan type I.

nate oligomers with different lengths. These unsaturated products are further degraded by the intracellular exolyase YesX to convert these oligomers to unsaturated disaccharides (13). The final step is the release of a single unsaturated galacturonate (5-dehydro-4-deoxy-D-galacturonate) by YesR and YteR (11). The RG-I side chains, including galactan, arabinan, and arabinogalactan, are dissociated from the backbone by two extracellular arabinan endo-1,5- α -L-arabinosidases, namely, AbnB (14) and AbnA (7, 15), releasing the α -L-arabinofuranoside residues as well as galactans from the arabinogalactan branched chains of RG-I. These enzymes provide oligoarabinans, which can be taken up via the AraNPQ transporter. Afterward, the intracellular oligoarabinan can be further degraded into arabinofuranose by α -L-arabinofuranosidases AbfA and Xsa and finally delivered to the pentose phosphate pathway by the *araABDLMN PQ-abfA* cluster (14, 16).

In addition to arabinan and arabinogalactan, galactan chains are also released during the degradation of pectin. In *Bacillus subtilis*, an extracellular enzyme, named galactanase GalA, was found to be able to degrade the galactan portion of arabinogalactan (17). Studying the glycoside hydrolases (GHs) of *B. subtilis* revealed an arrangement of a GH family 42 β -galactosidase gene, *lacA*, neighboring a GH family 53 enzyme *galA* gene. Cloning the two genes enabled *Escherichia coli* to grow with galactan as a carbon source (18). The previously mentioned GalA was later annotated as GanB (arabinogalactan endo- β -1,4-galactosidase) and belongs to the pentacistronic operon *cycB-ganPQAB*, which is regulated by GanR (also known as LacR) located upstream of this operon (19, 20). CycB (renamed GanS) is a sugar-binding lipoprotein that is reported to bind cyclodextrin (21) and forms the ABC transporter together with GanP and GanQ (22). Finally, the *ganA* gene (also known as *lacA*) encodes a β -galactosidase that is able to degrade substrates, such as *ortho*-nitrophenyl- β -galactopyranoside (oNP- β -Gal) or 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (18, 19). Prior to this study, the importance of the *cycB-ganPQAB* operon for the growth of *B. subtilis* with galactan was

shown (23). In this study, we characterized the function of GanB and GanA and their substrate specificity and thereby expanded our knowledge of galactan degradation by *B. subtilis*. Beside this, the uptake of the products and the specific inducer of the galactan utilization system were investigated.

MATERIALS AND METHODS

Strains, media, and growth conditions. All strains used in this study are listed in Table S1 in the supplemental material. *Escherichia coli* JM109 was used for plasmid propagation and gene expression throughout this study. *E. coli* strains containing the desired plasmids were selected on LB plates supplemented with ampicillin (100 μ g/ml) or spectinomycin (100 μ g/ml) depending on the plasmid selection marker. To overexpress the desired genes in *E. coli*, LB medium with ampicillin (100 μ g/ml) was inoculated with an overnight culture of *E. coli* carrying the target plasmid with a starting optical density at 600 nm (OD₆₀₀) of 0.05. After 2 h of incubation at 37°C with shaking at 200 rpm, 0.2% L-rhamnose was added to the bacterial culture at an OD₆₀₀ of about 0.3, and the cells were harvested by centrifugation after 4 h of incubation at 30°C. The cell pellet was kept at -20°C for further studies.

Bacillus subtilis KM0 (strain 168 with a functional TrpC) was used as the wild-type (wt) strain during this study. *B. subtilis* knockout strains (BKE) were provided by *Bacillus* Genetic Stock Center (BGSC; Columbus, OH). *B. subtilis* transformants were selected on LB plates containing spectinomycin (100 μ g/ml) or erythromycin (5 μ g/ml).

The tryptophan auxotroph strains were cultivated on Spizizen minimal plates (24) containing 50 μ g/ml tryptophan. To investigate the regulation of the galactan utilization genes *in vivo*, *B. subtilis* strains were inoculated to 40 ml LB medium with a starting OD₆₀₀ of 0.05. After 2 h of incubation at 37°C, 0.2% lupin galactan or 0.2% glucose (alone or in combination) was added to the bacterial culture, and the GanA β -galactosidase and GanB galactanase activities of each sample were measured after 6 h of cultivation at 37°C. All experiments were carried out in triplicate, and the mean values were used for further analysis.

Modification of *B. subtilis* knockout mutants. The *B. subtilis* knockout strains (BKE strains) provided by *Bacillus* Genetic Stock Center (BGSC) contained an erythromycin resistance gene flanked by *loxP* sites. In order to remove this antibiotic marker gene, the BKE strains were

transformed with pJOE6732.1 expressing Cre recombinase under the control of a constitutive P_{xyI} promoter originating from *Bacillus megaterium* QM B1551. After resolving the *loxP* sites, the antibiotic-sensitive strains were screened based on erythromycin or spectinomycin sensitivity. Plasmid pJOE6732.1 is derived from pAM β 1, which contains the minimal *repDE* genes, the replication origin without *copF*, the stabilizing genes *resB* and *topB*, and partition and toxin-antitoxin genes (25). Due to its high instability, pJOE6732.1 can easily be cured. To repair the *trpC2* mutation in the desired strains, plasmid pKAM041, containing the functional *trpC* gene, and its flanking regions were used.

Measurement of the GanB galactanase activity. Different substrates were used to measure the β -galactanase activity. As a natural substrate, arabinogalactan from larch wood (Sigma-Aldrich, Munich, Germany) or galactan from lupin, which was treated with arabinofuranosidase (Megazyme, Wicklow, Ireland), was used. The enzymatic reaction was carried out by adding appropriate amounts of enzyme (crude extracts or purified His₆-GanB) to 1% galactan in a total volume of 40 μ l potassium phosphate buffer (0.1 M, pH 6.5). After incubation at 37°C, samples were withdrawn, and the reaction was stopped by heating for 10 min at 95°C. The products of the enzymatic reactions were further qualitatively analyzed by thin-layer chromatography or by high-performance anion-exchange chromatography (HPAEC). In order to calculate the GanB activity in units, the reduced sugars released from the lupin galactan degradation were determined by the 3,5-dinitrosalicylic acid (DNS) assay (26). Briefly, appropriate amounts of His₆-GanB (0.5 μ g) in 40 μ l of potassium phosphate buffer (0.1 M, pH 6.5) were mixed with 40 μ l of 2% lupin galactan. The reaction mixture was incubated for 15 min at 37°C, and the reaction was stopped with 300 μ l dinitrosalicylic acid reagent. After heating at 95°C for 20 min, 1 ml H₂O was added and kept for 20 min on ice. The absorption of the developed orange color was measured at 540 nm. One unit was defined as the release of 1 μ mol of reducing sugar (here galactose) per minute determined by the DNS assay.

A standard GanB activity assay was performed with azurine-cross-linked-potato (AZCL) galactan (product 38127; Sigma-Aldrich, Munich, Germany) as the substrate in a colorimetric assay. The enzyme reaction was carried out with 0.2% AZCL galactan in a total volume of 250 μ l containing variable amounts of enzyme. The reaction mixture was incubated for 15 min at 37°C and stopped with 250 μ l of 0.4 M sodium borate. After centrifugation for 5 min at 13,000 \times g, 100 μ l of the blue colored supernatant was diluted with 900 μ l H₂O, and its absorbance was measured at 595 nm. As the blank, the reaction mixture ingredients without enzyme were used.

In order to test the degradation of galactotetraose, galactotriose, and galactobiose by GanB, appropriate amounts of purified GanB were incubated with these substrates, with the final concentration of 5 mM in a total volume of 10 μ l. The product composition of the reaction mixture was analyzed by high-pressure liquid chromatography (HPLC).

Measurement of β -galactosidase activity. β -Galactosidase activity was determined by measuring the rate of *p*-nitrophenyl- β -D-galactopyranoside (*p*NP- β -Gal) hydrolysis as described earlier (27). The β -galactosidase activity toward lactose was calculated from the amount of released glucose according to Placier et al. (27). One unit of the enzyme activity was defined as the release of 1 μ mol of *p*NP or glucose per minute, respectively. The substrates, galactotetraose, galactotriose, and galactobiose, were used with the final concentration of 5 mM in a total volume of 10 μ l. The product composition was analyzed by HPLC as described before. Finally, all β -galactosidase assays were carried out at 37°C using 0.1 M potassium phosphate buffer (pH 6.5).

Analysis of galacto-oligosaccharides by chromatographic methods. Qualitative and quantitative chromatographic methods were used for the analysis of galacto-oligosaccharides generated after the degradation of galactan. In the qualitative analysis, the separation of galacto-oligosaccharides was performed using thin-layer chromatography (TLC) on Silica Gel 60 F₂₅₄ plates (Merck, Darmstadt, Germany). The mobile phase was a mixture of acetone-H₂O (87:13, vol/vol). Carbohydrate spots were visu-

alized by soaking the TLC plates with a solution containing 10.5 g ammonium sulfate, 0.5 g cerium sulfate, and 15 ml concentrated H₂SO₄ in 245 ml H₂O. The carbohydrate blue spots were developed after backing the plates at 80°C. Quantitative analysis of individual galacto-oligosaccharide fractions was performed using high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (ESA Coulochem III; ESA Inc., Chelmsford, MA). The samples were separated on a CarboPac PA1 column (Dionex, Idstein, Germany) using the mobile phase of 0.22 M NaOH and 0.02 M sodium acetate with a flow rate of 0.75 ml/min as described previously (28). Individual carbohydrates, such as galactose, lactose, and β -1,4-galactobiose (β -D-Gal-[1 \rightarrow 4]- β -D-Gal) (product G9662; Sigma, Munich, Germany), were used as standards during HPLC analysis.

Isolation of galacto-oligosaccharides. Galacto-oligosaccharides used as a substrate for enzymatic reactions or in mass spectrometry analysis were isolated after enzymatic digestion of lupin galactan by the purified GanB enzyme in a preparative approach. One percent lupin galactan was treated with 12 μ g purified His₆-GanB in 0.1 M potassium phosphate buffer (pH 6.5) in a total volume of 800 μ l. The reaction was stopped after 30 min of incubation at 37°C by heating for 10 min at 95°C. After centrifugation for 10 min at 13,000 \times g, 400 μ l of supernatant was loaded onto a Bio-Gel P-2 column (250 ml; Bio-Rad, Munich, Germany) and separated with double-distilled water (ddH₂O) as the mobile phase. The 1-ml fractions were collected after a flowthrough of 70 ml and analyzed by HPLC. Fractions 15 to 21 were pooled as P1, fractions 26 to 31 were pooled as P2, and fraction 37 to 41 were pooled as P3, respectively. The P1, P2, and P3 samples were lyophilized prior to quadrupole time of flight liquid chromatography mass spectrometry (Q-TOF-LC/MS) analysis.

Methods in supplemental materials. DNA manipulation and plasmid construction, protein purification, DNA sequencing, electrophoretic mobility shift assay, DNase I footprinting, primer extension, and thermal shift assay methods are explained in the supplemental material.

RESULTS

GanB degrades arabinose-free galactan with its endo- β -1,4-galactosidase activity. To study the galactan utilization in *B. subtilis*, the characteristics and function of GanB encoded by the last gene of the *cycB-ganPQAB* operon were primarily investigated. GanB (also known as GalA or YvfO) is annotated as arabinogalactan endo- β -1,4-galactosidase, which belongs to glycoside hydrolase (GH) family 53. Analysis of the GanB protein sequence with the SignalP 4.1 program (29) predicted a signal peptide of 26 amino acid residues (data not shown). To further characterize the activity of GanB, the N-terminal His₆-tagged mature protein was produced in *E. coli* strain JM109(pHWG1119) and purified by immobilized-metal affinity chromatography (IMAC). The purification of His₆-GanB from the crude extract yielded over 90% pure His₆-GanB, with a molecular mass of 46 kDa and a protein content of 0.6 mg/ml. To characterize GanB, different substrates and methods were applied in the enzyme assay (see Table S3 in the supplemental material). To begin with, AZCL galactan, which is a dyed substrate, was used for a semiquantitative assay. By doing this, the optimal temperature of activity was determined to be 50°C and the optimal pH was 6.5 (data not shown). Also, the specific activity of GanB toward galactan that originated from lupin was determined by measuring the increase of reducing sugars during the enzymatic reaction and resulted in 75 \pm 5 U/mg GanB activity.

Given that galactan is degraded by GanB, in order to find out the possible mode of action of GanB, arabinogalactan from larch wood and lupin galactan were qualitatively tested by TLC analysis. After degradation of lupin galactan by GanB, galactose and galacto-oligosaccharides were produced. The latter products diminished after a longer period of degradation, and the proportion of

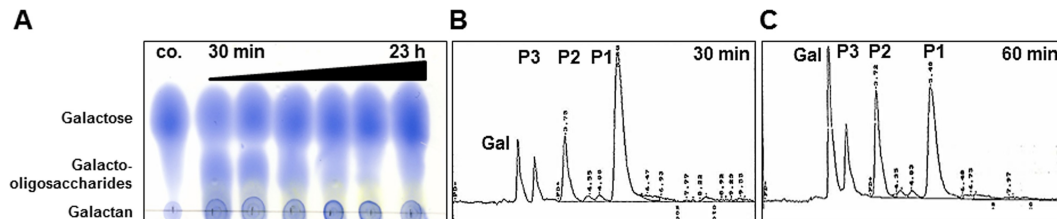


FIG 2 (A) Analysis of the products of lupin galactan degradation by GanB after 30 min and up to 23 h using thin-layer chromatography. As a control (co.), a mixture of galactose (20 μ g) and galactan (5 μ g) was applied. (B and C) HPLC profiles of the products of lupin galactan degradation by purified GanB after 30 min (B) and 60 min (C). The separation of the galacto-oligosaccharides was performed by HPLC with pulsed amperometric detection as described in Materials and Methods.

galactose increased (Fig. 2A). No product was released from arabinogalactan (data not shown). This showed that arabinose-free galactan is the natural substrate for GanB. In order to identify the galacto-oligosaccharides generated during the degradation of lupin galactan by GanB, the reaction mixture was further analyzed by HPLC (Fig. 2B and C). Two products, i.e., galactose and β -D-Gal-(1 \rightarrow 4)- β -D-Gal (here Gal₂ or galactobiose), were identified using commercial standards (Fig. 2B and C). Additionally, two further unidentified main products (shown as P2 and P1 in Fig. 2) were obtained with a higher degree of polymerization (DP) and retention times of 3.78 and 5.49, respectively (Fig. 2B and C). The proportion of these galacto-oligosaccharides with higher DP decreased after a longer incubation of lupin galactan with GanB (Fig. 2B and C).

In order to characterize the unknown galacto-oligosaccharides generated during the galactan degradation, a preparative approach was applied. First, the reaction mixture of the degradation of 4 mg lupin galactan was separated on a Bio-Gel P-2 chromatography column, and products with different DP were isolated (labeled as P1, P2, and P3 fractions) (Fig. 3A and B). Afterward, their molecular masses were determined by Q-TOF-LC/MS with a negative-ion [M-H]⁻ mode (Fig. 3C). Mass spectrometry analysis revealed that P1 was a tetrasaccharide with a molecular weight of

666.2227, P2 was a trisaccharide with a molecular weight of 504.1688, and P3 was a disaccharide with a molecular weight of 342.1161 (Fig. 3C). The latter substance has also been identified as β -D-Gal-(1 \rightarrow 4)- β -D-Gal with an authentic standard by HPLC. These experiments show that GanB cleaves the high-molecular-weight arabinose-free galactan polymer producing galacto-oligomers with a DP of up to 4 and provide evidence for the endo-type mode of action.

GanB was also subjected to substrates other than high-molecular-weight galactan in order to determine the GanB mode of action. First, the isolated galactotetraose, galactotriose, and galactobiose served as GanB substrates, and the degradation products were analyzed by HPLC (Fig. 4). In all of these cases, the release of galactose was detected, suggesting that GanB somewhat shows an exo-mode of action. However, GanB was unable to act on *p*-nitrophenyl- β -D-galactopyranoside (*p*NP- β -Gal), indicating that a solely exo-mode could not be detected (see Table S3 in the supplemental material). All in all, GanB shows both endo- and exo-activity, with galactan as a substrate generating galactotetraose as its main product.

Galactotetraose and galactotriose are transported by galactan ABC transport system (GanSPQ-MsmX). As shown before, degradation of galactan by GanB generated galacto-oligosaccha-

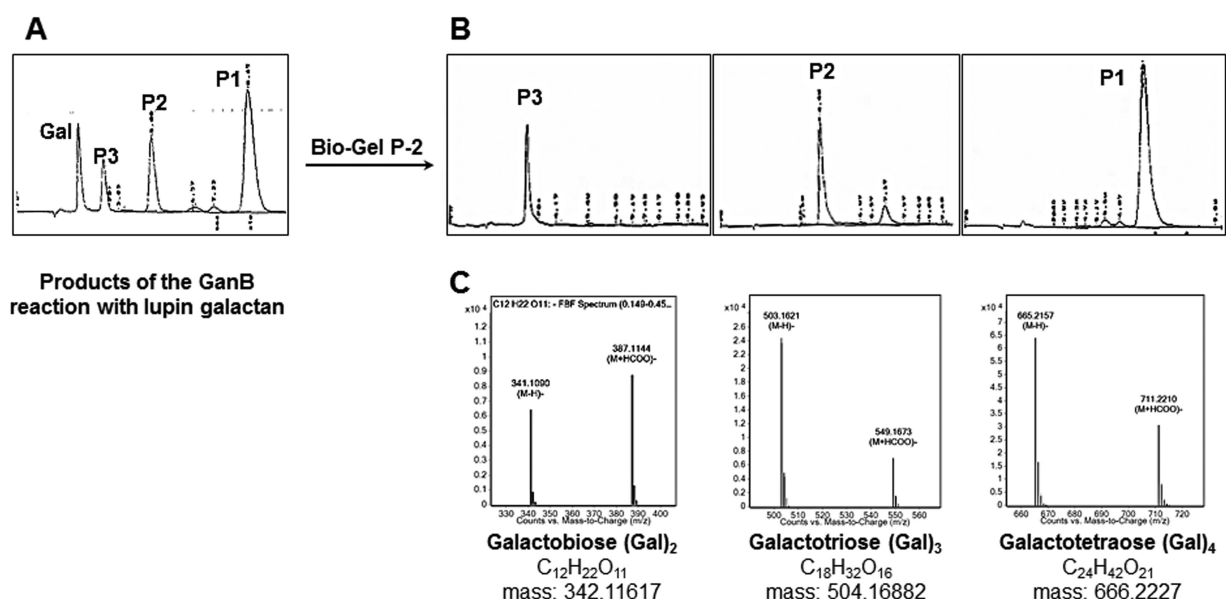


FIG 3 (A) HPLC profile of products from the preparative degradation of galactan with GanB. (B and C) HPLC chromatograms of the galacto-oligosaccharides P1 to P3 that were isolated after Bio-Gel P-2 chromatography (B), along with their mass spectra peaks elucidated by Q-TOF-LC/MS (C).

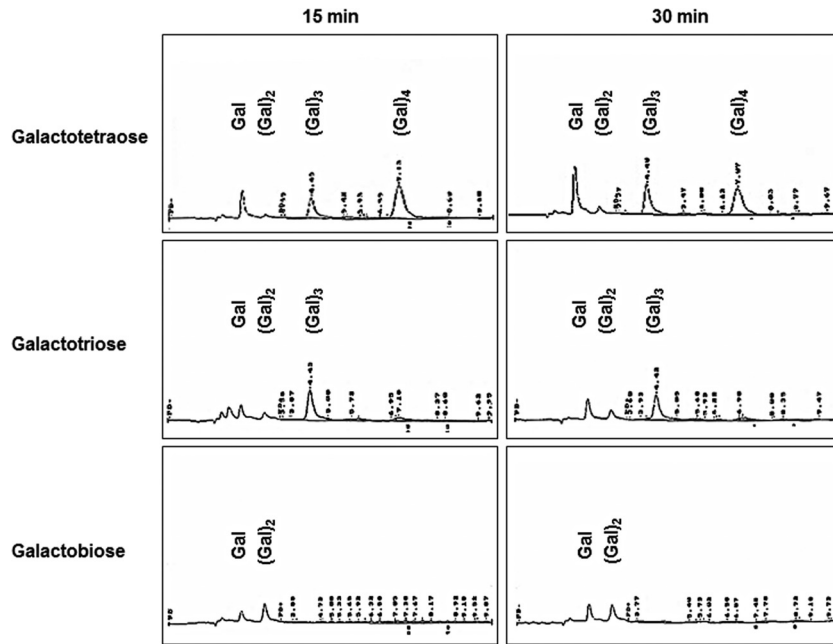


FIG 4 HPLC profile of the degradation of galactotetraose, galactotriose, and galactobiose by GanB after reaction times of 15 and 30 min to demonstrate the exo-mode of action of GanB. The separation of the galacto-oligosaccharides was performed by HPLC with pulsed amperometric detection as described in Materials and Methods.

rides with a DP of up to 4. To clarify the pathway of galactan utilization, the next step was to investigate the substrate specificity of the galactan transporter. The galactan transporter is an ABC transport system, consisting of GanP and GanQ as the transmembrane proteins and CycB as the substrate-binding lipoprotein. The substrate specificity of the transporter depends on CycB, which has been shown to be a cyclodextrin-binding lipoprotein (21). Analysis of the CycB protein sequence indicated an LAAC lipoprotein signal peptide motif (30) with a cleavage site between amino acid positions 22 and 23 (data not shown). To carry out the *in vitro* studies, the mature CycB was fused to the His₆ tag at its N terminus, and the His₆-CycB was overproduced in *E. coli* using the plasmid pHWG1151. SDS-PAGE analysis of the crude extract of induced JM109(pHWG1151) showed the production of the 44-

kDa His₆-CycB protein. Purified His₆-CycB (2.5 mg/ml) was used for the thermal shift assay (Fig. 5A). In this assay, CycB was subjected to three substrates, namely, galactobiose, galactotriose, and galactotetraose, which are produced by the activity of GanB. Only galactotetraose and galactotriose were able to increase the melting temperature of CycB by their binding to His₆-CycB (Fig. 5A). Additionally, α - and β -cyclodextrins were tested with the thermal shift assay. Although cyclodextrin was supposed to be the ligand of CycB (21), no affinity of cyclodextrin to His₆-CycB was observed under the experimental conditions (Fig. 5A). Therefore, we renamed CycB to GanS here. Altogether, this experiment shows that galactotetraose and galactotriose are the main ligands for the GanS protein and may be mainly transported by the galactan ABC transporter into the cytoplasm.

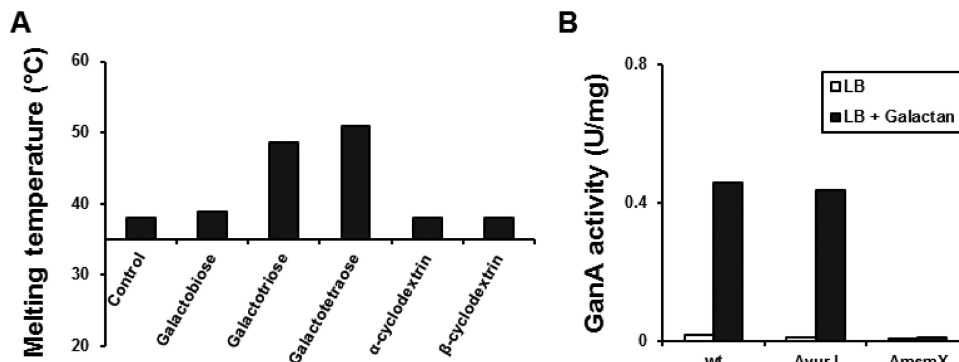


FIG 5 (A) Thermal shift assay using purified His₆-CycB (later renamed GanS) in the presence of galactobiose, galactotriose, galactotetraose, α -cyclodextrin, and β -cyclodextrin with a final concentration of 1 mM. The melting temperature of His₆-CycB was measured without the substrate (control). (B) Finding the ATP-binding protein of the galactan ABC transporter. The wild-type strain BKE32550 ($\Delta yurJ$) and BKE38810 ($\Delta msrX$) were cultivated in LB with or without 0.2% lupin galactan with a starting OD₆₀₀ of 0.05 and were induced after 2 h of incubation at 37°C. The β -galactosidase activity of GanA was measured in crude extracts after 6 h of induction.

Another important question about the uptake of the galacto-tetraose and galactotriose by the galactan ABC transporter was the ATP-binding protein. Since the *ganSPQAB* operon contained no ATP-binding protein, the genome of *B. subtilis* was searched for the probable ATP-binding proteins. So far, 70 ATP-binding proteins have been annotated and predicted in the genome of *B. subtilis*, among which 2 proteins, namely, MsmX and YurJ, are suggested to be multiple sugar-binding transporter ATP-binding proteins (18). Therefore, two strains lacking *msmX* (strain BKE38810) or *yurJ* (strain BKE32550) were cultivated in LB with or without galactan as an inducer. As a reporter system, the β -galactosidase activity of GanA in the crude extract was measured (Fig. 4B). In the wild-type strain, the β -galactosidase activity was increased by 24-fold when galactan was added to LB medium. Deletion of *yurJ* had no influence on the β -galactosidase activity, showing similar inducibility to the wild-type strain. On the contrary, adding galactan to LB had no influence on the β -galactosidase activity in the $\Delta ms m X$ mutant. The loss of induction of the *gan* operon in the $\Delta ms m X$ mutant is caused by a galacto-oligosaccharide transport deficiency. Taken together, MsmX is the ATP-binding component of the galactan ABC transport system.

GanA degrades galacto-oligosaccharides in an exo-type mode of action. The *gan* operon contains another structural gene, *ganA* (also known as *lacA* or *yvfN*), which encodes a β -galactosidase. The activity of this enzyme toward the synthetic substrate oNP- β -Gal was shown several years ago (20). Nevertheless, by knowing the mechanism of the extracellular degradation of galactan and the uptake of the produced galacto-oligosaccharides, it was essential to understand the degradation of these galacto-oligosaccharides by GanA. Therefore, the *his₆-ganA* gene was over-expressed in *E. coli* using strain JM109(pHWG1111). The production of His₆-GanA was confirmed by SDS-PAGE and showed a prominent protein band with a molecular mass of 80 kDa (data not shown). The purified His₆-GanA exhibited a specific activity of 80 U/mg toward the synthetic substrate pNP- β -Gal at 37°C. Using pNP- β -Gal as the substrate, no metal dependency was observed, and GanA exhibited an optimum pH and temperature of 6.5 and 55°C, respectively (data not shown). Likewise, GanA was able to cleave lactose with a specific activity of 12 U/mg.

Finally, the target substrates of the galactan utilization system were tested for GanA activity (see Table S4 in the supplemental material). At first, the high-molecular-weight substrates were used as the substrates for GanA. For this purpose, different methods were used to detect the degradation products of arabinogalactan, AZCL galactan, and lupin galactan. In all of these cases, no GanA activity was detectable (see Table S4). Conversely, the galacto-oligosaccharides, galactotetraose, galactotriose, and galactobiose, were degraded by GanA generating galactose as the end product. The HPLC degradation profile of galactotetraose is shown in Fig. 6. Altogether, these results clearly indicated that GanA degrades the transported galacto-oligosaccharides into galactose as the end product. Since galactose was the main end product of the degradation of galactotetraose, it is highly assumable that GanA act as an exo-saccharolytic enzyme. Furthermore, it should be noted that the activity for galactotetraose was approximately 20-fold higher than that for lactose with a 5 mM substrate in an activity test (data not shown).

Induction of the *ganSPQAB* operon by galactobiose and its repression by glucose. The galactan utilization operon is repressed by GanR (19); however, the possible inducer of this system

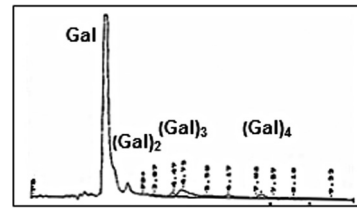


FIG 6 Degradation of prepared galactotetraose by GanA. The reaction was carried out for 15 min at 37°C.

has not yet been reported. In order to explore the possible inducer of GanR, wild-type *B. subtilis* and $\Delta ganR$, $\Delta ganS$, $\Delta ganA$, and $\Delta ganB$ mutant strains were cultivated in LB containing galactan or glucose alone or in combination, and their galactanase activities (GanB) were measured in the culture medium (Fig. 7A). The wild-type strain cultivated in LB with galactan showed significantly higher GanB activity (5-fold) than the wild-type strain cultivated in LB without galactan. This shows that the presence of galactan induced *ganB* expression. Similar to many carbohydrate utilization systems in *B. subtilis*, the addition of glucose to the culture medium reduced GanB activity regardless of the presence of galactan, showing that the galactan utilization system is repressed by carbon catabolite repression. Deletion of GanR caused a high level of *ganB* expression where the GanB activity with or without galactan was almost identical, albeit significantly higher than the induced wild-type strain. The carbon catabolite repression remained functional in the $\Delta ganR$ mutant. Deletion of *ganS* resulted in lower GanB activity when galactan was present in the medium, showing a lower induction of the system that was probably caused by the loss of inducer uptake. Interestingly, deletion of *ganA* increased GanB activity by 2.5-fold compared to that of the wild-type strain cultivated in LB with galactan. In this case, the inducibility of *ganB* was 4-fold. When *ganB* was deleted, negligible GanB activity was measured in the culture medium (Fig. 7A).

In order to confirm the results obtained by the measurement of the GanB activity, the GanA activity in the wild-type strain and in the $\Delta ganR$, $\Delta ganS$, $\Delta ganA$, and $\Delta ganB$ mutants was also determined (Fig. 7B). In this experiment, the *gan* system was inducible by galactan, while the *ganR* deletion rendered the *gan* operon constitutive. The *ganS* deletion significantly reduced GanA activity, which may be due to the reduced uptake of the probable inducer of the system (Fig. 7B). The *ganA* deletion showed negligible β -galactosidase activity as expected. Finally, when *ganB* was deleted, also no β -galactosidase activity was detected (Fig. 7B). Taken together, these results indicate that the inducer of the system is a galacto-oligosaccharide generated by GanB activity. Since deletion of *ganA* did not affect the induction of the system and even increased GanB activity in the presence of galactan (Fig. 7A), it is likely that the inducer of the system is one of the transported galacto-oligosaccharides.

To identify the inducer of the galactan utilization system, an electrophoretic mobility shift assay was conducted using the purified Strep-tagged GanR and different potential substrates (Fig. 7C). The 5'-end fluorescein isothiocyanate (FITC)-labeled P_{gan} DNA was amplified from the promoter region located upstream of *ganS*. From the addition of GanR to the P_{gan} DNA in the reaction mixture, a DNA-protein complex formed that migrated slower than the free P_{gan} DNA. Next, galactose or different ga-

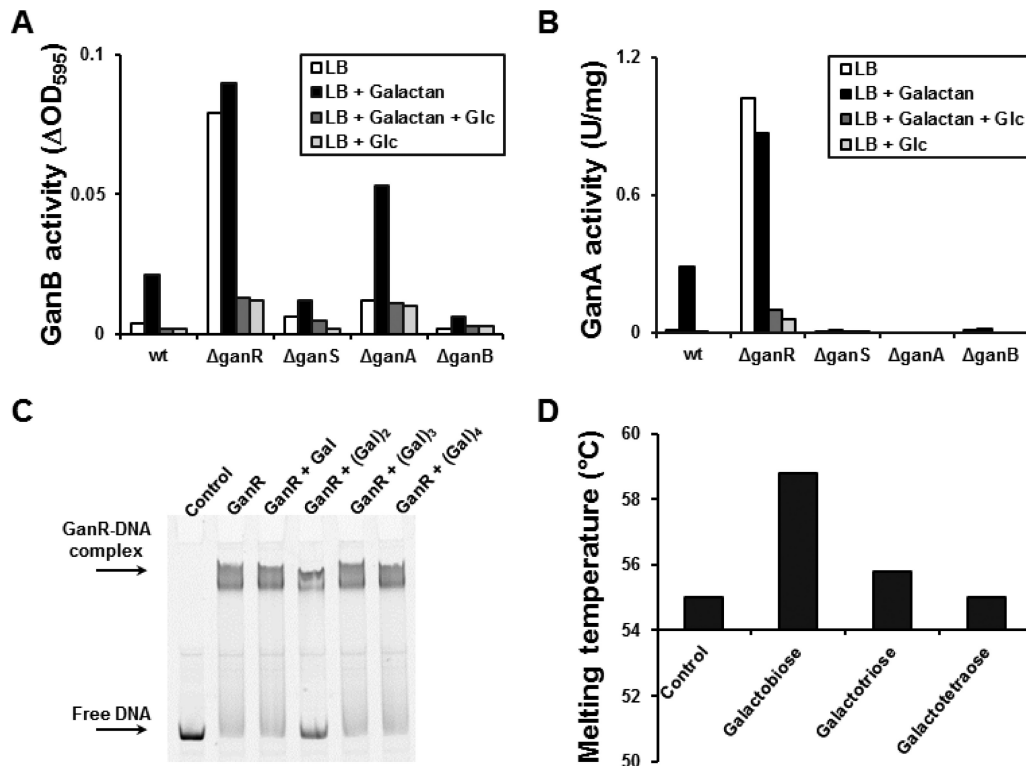


FIG 7 (A and B) GanB (A) and GanA (B) activities after 6 h of cultivation of *B. subtilis* strains, i.e., wt (KM0), Δ *ganR* (BKE34170), Δ *ganS* (KM468), Δ *ganA* (KM589), and Δ *ganB* (KM588), with different sugars in LB as described in Materials and Methods. GanB activity was measured in a 1:10 diluted culture medium toward the substrate AZCL galactan. GanA activity was determined in crude extracts toward pNP- β -Gal. (C) An electrophoretic mobility shift assay was carried out using the amplified 5'-end $Cy5$ - P_{gan} DNA in the absence (control) and presence of GanR. The potential inducers of GanR were added to the reaction with the final concentration of 5 mM. (D) Thermal shift assay using purified GanR-Strep tagged in the absence of substrate (control) and in the presence of galactose, galactobiose, galactotriose, or galactotetraose with a final concentration of 1 mM.

lacto-oligosaccharides were added to the DNA-protein complex. Only galactobiose was able to dissociate the GanR from its target P_{gan} DNA, showing that the galactobiose is the inducer of the galactan utilization system (Fig. 7C). Lactose and isopropyl- β -D-thiogalactopyranoside (IPTG) also had no influence on the DNA-protein complex (data not shown). To confirm this result, thermal shift assay was also carried out using the purified GanR and the galacto-oligosaccharides. Indeed, the presence of galactobiose increased the melting temperature of GanR (Fig. 7D). Altogether, galactobiose was found as the inducer of the *gan* system using *in vivo* and *in vitro* experiments.

GanR inhibits the P_{gan} transcription by binding to the -35 box. As the final step in this study, the promoter region of the *ganSPQAB* (P_{gan}) was characterized. The transcription start site of the *gan* operon was identified by primer extension method (Fig. 8A). The results of the primer extension experiment indicated that the transcription of *ganSPQAB* starts 38 bp upstream of the translation start codon of *ganS* (Fig. 8A). By the identification of the +1 position, the typical -10 (TATAAT) and -35 (TTtACA) boxes of the σ^A -type (housekeeping) promoters were found in the P_{gan} DNA sequence, each with a mismatch to the consensus sequence (small letters) (Fig. 8D). Interestingly, a TGN motif was found next to the -10 box showing that P_{gan} likely had an extended -10 box (Fig. 8D). Given that the P_{gan} is repressed by GanR, DNA footprinting was carried out using purified Strep-tagged GanR (see Fig. S1 in the supplemental material). Digestion of the P_{gan}

DNA by DNase I indicated a protected region between nucleotides -14 and -79 with respect to the transcription start site at the coding strand (Fig. 8B), whereas the noncoding strand was protected between bp -20 and -75 from the transcription start site (Fig. 8C). These results show that GanR represses the P_{gan} transcription initiation via the steric hindrance mechanism and by prevention of the binding of the RNA polymerase.

DISCUSSION

Galactan is an abundant molecule in the cell wall of plants and obviously a suitable substrate for the soil bacteria as an important source of galactose. For the first time, the galactanase activity of *B. subtilis* was detected by its cultivation in a medium containing soybean. This galactanase was able to degrade β -1,4-galactan into the predominant product galactotetraose (17). The galactanase-encoding gene, *gala* (later renamed *ganB*), was discovered in *B. subtilis* after its importance for the degradation of galactan by *E. coli* was proven *in vivo* (18). In this study, we provided *in vitro* evidence that GanB, which belongs to GH family 53 of the β -1,4-galactanases, indeed acts as a β -galactanase. Moreover, GanB exhibits an endo- and exo-mode of action producing galactotetraose as the main product along with galactotriose, galactobiose, and galactose (Fig. 3). Similar patterns of product release were reported from the β -galactanase GanA from *Geobacillus stearothermophilus* (31) and the β -galactosidase from *Bacillus licheniformis* (BLGAL) (32). Nevertheless, there is a difference in the substrate

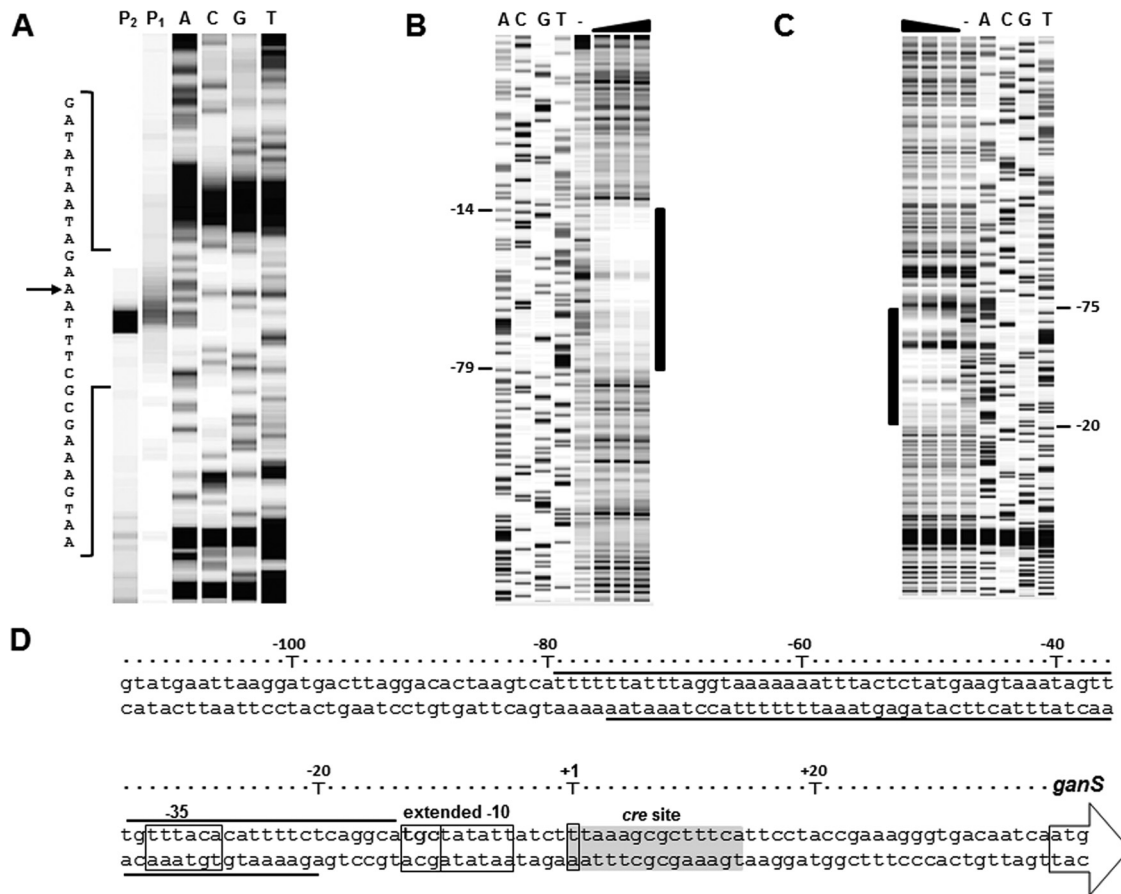


FIG 8 Characterization of the core and regulatory elements of P_{gan} . (A) Identification of the transcription start site of P_{gan} was carried out using primer extension reactions. The generated cDNA probes (P_1 and P_2 repeats) were compared with the sequencing reaction using the dideoxy chain-termination method (A, C, G, T). (B and C) The DNase I footprinting reactions for the P_{gan} coding strand DNA (B) and noncoding strand DNA (C) were compared with the dideoxy chain-termination reactions (A, C, G, and T). The DNase I footprinting reactions were performed without GanR as the negative control (–) or with different amounts of GanR (1.95, 3.90, and 7.79 μ M). The bar shows the protected DNA region in each DNase I footprinting reaction. (D) The DNA sequence of the P_{gan} region. The promoter core elements (–35 box and extended –10 box) and the transcription start site (+1) are shown by rectangles. The protected DNA region is shown by solid lines. The arrow shows the start codon of *ganS*. The predicted *cre* site is highlighted in gray.

preference of these enzymes. While GanB is able to degrade galactotetraose, galactotriose, and galactobiose, suggesting an additional exo-mode of activity (Fig. 4), BLGAL is not able to hydrolyze galactotriose and galactobiose to any appreciable extent although their amino acid sequences are 80% identical. This sequence identity between GanB and BLGAL even includes three homologous tryptophan side chains that are involved in galactotriose binding (32). Compared to galactanases from *Bacilli* species, fungal β -galactanases perform a more complete degradation of galactan, producing mostly galactose and galactobiose as end products (32, 33). The liberation of galactotriose as the sole product in an exo-fashion was reported from an endo- β -galactanase from *Bifidobacterium longum* (34). These results support the idea that different types of product formation exist among GH family 53 galactanases.

After degradation of galactan by the extracellular GanB, the released galactotetraose, galactotriose, and galactobiose must be transported into the cell. The galacto-oligosaccharide transport system is one of the 10 carbohydrate-specific ABC transport systems in *B. subtilis* (35) with two transmembrane proteins, GanP and GanQ, and a substrate-binding protein, CycB. The annota-

tion of CycB originates from a previous report on the ability of CycB to bind β - or γ -cyclodextrin (21). Here, we provided evidence that CycB is clearly a galacto-oligosaccharide-binding protein, and it showed no affinity toward β -cyclodextrin as a substrate under our experimental condition. Therefore, we renamed *cycB* to *ganS* as a substrate-binding lipoprotein. The next unknown component of the galacto-oligosaccharides transport system was the ATP-binding protein. Previously, Shipkowsky and Brenchley (18) proposed that one of the two MalK homologs, namely, YurJ and MsmX proteins, is the possible ATPase of the galactan ABC transporter. Our results indeed showed that MsmX is the ATPase of the galactan ABC transporter. In addition to the galactan ABC transport system, MsmX energizes the AraNPQ (arabino-oligosaccharides) (36) and MdxEFG (maltodextrin) (37) ABC transport systems.

The transported galactotetraose and galactotriose are further degraded in the cell. The *ganB* gene is flanked by *ganA*, which encodes a GH family 42 β -galactosidase. Interestingly, lactose, which is probably the natural substrate for β -galactosidases (38), is weakly degraded by GanA (see Table S4 in the supplemental material). Similar to *B. subtilis* GanA, GanB of *G. stearothermophi-*

lus shows no detectable activity toward lactose (31). In *B. subtilis*, the physiological role of GanA is to hydrolyze the transported short β -1,4-galacto-oligosaccharides into galactose, while it is unable to degrade the galactan polymer similar to GanB of *G. stearothermophilus* (31). The arrangement of GH family 53- and GH family 42-encoding enzymes within the galactan utilization cluster was already reported for *Geobacillus stearothermophilus* (31). It seems a common feature that the degradation of galactan in nature is carried out by an extracellular endo- β -galactanase that cleaves the high-molecular-weight polysaccharide inside the chain and produces short oligomers. Afterward, the intracellular β -galactosidase enzyme GH family 42 hydrolyzes these short galactooligosaccharides into galactose. Galactose can then be metabolized into UDP-glucose via the Leloir pathway (23).

Transcriptome analysis of *B. subtilis* shows that the *ganSPQAB* genes form an operon (39), which is repressed by GanR (19). Prior to this study, the inducer of the system was not reported. Here, we found out that galactobiose is the inducer of the operon. This was in line with *in vivo* findings that the presence of the substrate-binding lipoprotein, GanS, and the extracellular GanB is necessary for the induction of the system. In contrast, the deletion of *ganA* even increased the inducibility of the *gan* operon and raised its maximal expression level in the presence of galactan (see Fig. 7A). This suggests that GanB extracellularly produces galactobiose from galactan, and GanS takes up galactobiose while GanA activity results in the degradation of galactobiose. Interestingly, the maximal activity of P_{gan} in the induced Δ *ganA* mutant was higher than that in the induced wild-type strain. This is probably due to the deficiency of the Δ *ganA* mutant to consume the galactobiose as a carbon source. Conversely, this maximal P_{gan} activity in the Δ *ganA* strain remained less than that in the Δ *ganR* mutant for two reasons. First, GanB mainly generates galactotetraose and, only after longer incubation times, galactobiose. Second, GanS has a lower affinity toward galactobiose than galactotetraose (or galactotriose) for the uptake. Nevertheless, it seems that the amount of galactobiose in the Δ *ganA* mutant is enough for the induction of P_{gan} . Finally, the characterization of the P_{gan} elements revealed that GanR represses the binding of the RNA polymerase with the housekeeping sigma factor by the steric hindrance mechanism. In addition, the *gan* operon is repressed in the presence of glucose similar to other carbohydrate utilization systems in *B. subtilis* (40). A potential *cre* (catabolite responsive element) site was found at position +1 (Fig. 8D). The *cre* sites are the specific binding sites of the carbon catabolite protein A (CcpA)-histidine-containing protein (HPr) complex, which acts as the general carbon catabolite repression system (41). The CcpA-HPr forms as a response to the high level of fructose 1,6-bisphosphate and glucose 6-phosphate in the cytoplasmic pool, which increases during the presence of preferred sugars, such as glucose or fructose (42). Depending on the location of the *cre* sites, binding of the CcpA-HPr complex results in the activation (upstream of -35 box) or repression (downstream of -10 box) of the system (40). In the case of the *gan* operon, the location of the *cre* site, which is downstream of the -10 box, results in blocking the transcription initiation of the *gan* operon in the presence of glucose.

Based on the results obtained in this study, the model of the galactan utilization system by *B. subtilis* is demonstrated in Fig. 9. In the first step, extracellular galactan is degraded by GanB generating mainly galactotetraose as well as galactotriose and galactobiose. Next, galactotetraose and galactotriose are transported via

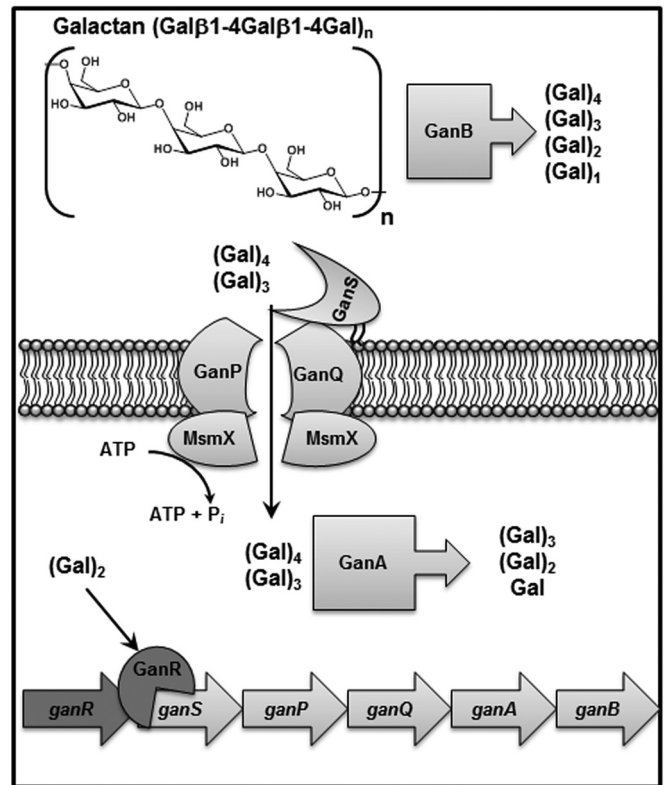


FIG 9 Model of the galactan degradation pathway in *B. subtilis*.

the GanSPQ-MsmX complex and further degraded by GanA inside the cell. Binding the galactobiose to GanR dissociates the GanR- P_{gan} complex, resulting in expression of the *gan* operon.

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