Note

Comparative Study on the Ability to Produce Gentiobiose in Cellulose-producing Bacteria *Asaia bogorensis* and *Gluconacetobacter xylinus*

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Abstract: In a cellulose-producing bacterium *Gluconacetobacter xylinus*, endo-β-1,4-glucanase and β-glucosidase are suggested to have an important role in cellulose biosynthesis, and genes encoding these enzymes are present adjacent to the cellulose synthesis operon. However, in other cellulose-producing bacteria including *Asaia bogorensis*, β-glucosidase gene is not found next to the cellulose synthesis operon, and their cellulose productivities are lower than that of *G. xylinus*. To investigate whether *A. bogorensis* produces a similar type of β-glucosidase, crude enzyme fractions were prepared from *G. xylinus* and *A. bogorensis* The hydrolytic activities toward p-nitrophenyl-β-D-glycosides were compared. In *A. bogorensis*, the hydrolytic activities were found in the cytosolic fraction, but the fraction did not indicate an ability to produce gentiobiose that is suggested to be an inducer for expression of the endo-β-1,4-glucanase gene in *G. xylinus*. We conclude that the mechanism of cellulose biosynthesis regulated by endo-β-1,4-glucanase may be different in *A. bogorensis* and *G. xylinus*.

Key words: *Asaia bogorensis*, β-glucosidase, gentiobiose, bacterial cellulose, cellulose biosynthesis

A number of bacterial species are known to produce cellulose, and bacterial cellulose holds promise for unique applications in various industries. Among the cellulose-synthesizing bacteria, *Gluconacetobacter xylinus* produces abundant amounts of cellulose and this member of the acetic acid bacteria (AAB) has been studied extensively as a model organism for cellulose biosynthesis. Cellulose is synthesized in membrane protein complexes that are organized as linear or rosette complexes in cellulose-synthesizing organisms and are referred to as terminal complexes (TCs). *G. xylinus* has a linear TC and each cellulose-synthesizing site in this complex is made up of a number of proteins. Cellulose synthase (CesA) and other proteins that possibly associate with the cellulose synthase are encoded for by the cellulose synthesis operon. The cellulose synthesis operon in *G. xylinus* encodes for three (CesAB, CesC and CesD) or four proteins (CesA, CesB, CesC and CesD),1,2 depending on the bacterial strains, and genetic analysis has demonstrated a role for all these proteins in cellulose biosynthesis.2–3

A variety of β-linked oligosaccharides have been identified in the culture broth during cellulose production by *G. xylinus*.4 Among these oligosaccharides, gentiobiose, β,1,6-linked glucosidase was found to accumulated more than the others. Kawano et al. reported that β-glycosidase (Bgl, GH family 3, EC 3.2.1.21) catalyzed the synthesis of gentiobiose from glucose in the culture medium by a condensation reaction, and that gentiobiose then enhanced the activity of endo-β-1,4-glucanase (CMCase, GH family 8, EC 3.2.1.4) by inducing the expression of the CMCase gene (*cme*).5 As CMCase is known to influence cellulose production,6,7 gentiobiose accumulation could regulate it in *G. xylinus*. The genes for CMCase and β-glucosidase, *cme* and *bgl*, are located upstream and downstream of the cellulose synthesis operon in *G. xylinus*, respectively.8,9

*Asaia bogorensis* was a gram-negative and aerobic AAB. We demonstrated for the first time that *A. bogorensis* produces cellulose among AAB except for *G. xylinus*.10 *A. bogorensis* also produce a water soluble extracellular polysaccharide, β,2,6-linked levan-type fructan when sucrose was used as a carbon source.10 The cellulose producing-ability of *A. bogorensis* is much lower compared to that of *G. xylinus*, and the cellulose produced by *A. bogorensis* is composed of thinner fibrils having low crystallinity.10 A cellulose synthesis operon (GenBank: AB355706) has been identified in *A. bogorensis*, and a CMCase-encoding gene homologous to the *cme* gene is located upstream of this operon similar to what is observed in *G. xylinus*. However, no β-glucosidase-encoding gene homologous to the *bgl* gene of *G. xylinus* is

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Abbreviations: TC, Terminal complex; CMCase, Endo-β,1,4-glucanase from *Gluconacetobacter xylinus*; Bgl, β-Glucosidase from *Gluconacetobacter xylinus*; *cme*, CMCase gene; *bgl*, Bgl gene; AAB, Acetic acid bacteria; SAB, Sodium acetate buffer; Extracellular fraction; Cytosolic protein fraction; β-glucosidase; Endo-β-1,4-glucanase; *pNPG1*, *p-Nitrophenyl-β-D-glycosides; *pNP*, *p-Nitrophenyl-β-D-glucopyranoside; *pNP2*, *p-Nitrophenyl-β-D-cellotrioside; *pNP3*, *p-Nitrophenyl-β-D-cellotetraoside*; TEC, Thin-layer chromatography; HPLC, High performance liquid chromatography.
found adjacent and downstream of the cellulose synthesis operon in *A. bogorensis*. Many other cellulose-producing bacteria do not also show the presence of the β-glucosidase gene downstream of the cellulose synthesis operon as observed in *G. xylinus*. Interestingly, the cellulose producing ability of these bacteria including *A. bogorensis* that do not show the presence of the β-glucosidase gene adjacent and downstream of the cellulose synthesis operon, is quite low in comparison to *G. xylinus*. From these points of view, β-glucosidase activity, especially gentiobiose-forming activity among cellulose-producing bacteria should be compared. To determine if *A. bogorensis* has β-glucosidase activity similar to that observed in *G. xylinus*, protein fractions were prepared from cultured bacterial cells and culture medium, and β-glucosidase activity and the ability to catalyze the condensation reaction to produce gentiobiose were analyzed in each fraction.

Protein fractions for investigation of β-glucosidase activity were prepared using the following procedure. Precultures (3 mL) of *A. bogorensis* JCM10569 and *G. xylinus* ATCC23769 grown in test tubes were suspended separately in 100 mL of SH medium each containing 20 g/L glucose, 5.0 g/L yeast extract, 5.0 g/L peptone, 0.27 g/L Na₂HPO₄·12H₂O and 0.115 g/L citric acid in a 300 mL Erlenmeyer flasks. After incubation on a rotary shaker (115 rpm) for 72 h at 25°C, the cell suspension was centrifuged at 6,000 rpm for 10 min at 4°C to separate the cells in the pellet and the culture medium in the supernatant. The supernatant was treated with ammonium sulfate to 90% saturation for 12 h at 4°C, and the precipitate formed was collected by centrifugation at 13,000 rpm for 15 min at 4°C and dissolved in 50 mM sodium acetate buffer (SAB) (pH 5.5, 4 mL). The protein suspension was dialyzed overnight against 50 mM SAB (pH 5.5) at 4°C and used as an extracellular protein fraction (extracellular fraction). The cell pellet was suspended in 50 mM SAB (pH 5.5) and after washing twice with 50 mM SAB (pH 5.5) the cells were suspended in the same buffer (5 mL). The cell suspension was sonicated using Sonics vibra cell³⁹, model VCX-130 (Sonics 130 W/60 Hz. Sonics & Materials, Inc., Newtown USA) in an ice bath with an amplitude of 40% and using pulsed mode of 01:01 s on: off for different time intervals for 3 min. The sonicated cell suspension was centrifuged at 13,000 rpm for 15 min at 4°C to obtain a pellet and supernatant. The supernatant was used as a cytosolic protein fraction (cytosolic fraction). The pellet was washed twice with 50 mM SAB (pH 5.5), suspended in the same buffer (5 mL) and used as the insoluble fraction including membrane proteins (insoluble fraction). All protein fractions were freeze-dried to be used for subsequent experiments after dissolving or suspending in 20 mM SAB (pH 5.5).

For each fraction, β-glucosidase activity was determined using cellobiose and p-nitrophenyl-β-D-glucopyranoside as substrates.

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**Fig. 1.** Pattern of hydrolysis of cellobiose and pNPG1 using different protein fractions obtained from cultures of *G. xylinus* and *A. bogorensis*.

(a) The carbohydrate spots were detected by the sulfuric acid-baking method, and (b) p-nitrophenol spots were visualized under UV light. The two arrowheads indicate the fractions that hydrolyzed the substrates, and the additional spots predicted pNP-β-D-gentiobioside are indicated by arrows. Lane M, cellooligosaccharide standards (G1, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose); lane P, pNP-β-D-cellooligosaccharide standards (pNPG1, pNPG2, pNPG3, pNPG4). Lane ct indicates the reaction without substrate. Lanes g and p indicate lanes with cellobiose and pNPG1 in the reaction, respectively. Ext., extracellular fraction; Cyt., cytosolic fraction; Ins., insoluble fraction.
(pNPG1) as substrates. The enzyme reaction was performed at 40°C for 60 min in 20 mM SAB (pH 5.5) containing 10 mM substrate (cellobiose or pNPG1) and 50 μg/mL protein fractions in 50 μL-reaction volume. The reaction products were detected by thin-layer chromatography (TLC) using a Silica Gel 60 Å plate (Whatman Ltd., Kent, UK) and mixture of chloroform-methanol-water (90:65:15) as the solvent. At these reaction conditions, no hydrolysis of cellobiose was observed for any protein fraction of both G. xylinus and A. bogorensis (Fig. 1). It is reported that Bgl from G. xylinus hydrolyzed cellobiose only slightly, although it produces glucose from cellobiose and larger cellobiosaccharides.13 We did observe hydrolysis of pNPG1 when the insoluble fraction of G. xylinus and the cytosolic fraction of A. bogorensis were used in the reaction. Both fractions hydrolyzed pNPG1 to glucose and p-nitrophenol. However, the products obtained using these two fractions were found to be slightly different, and an additional spot was detected at the position between p-nitrophenyl-β-D-cellotrioside (pNPG2) and p-nitrophenyl-β-D-cellotetraoside (pNPG3) in the insoluble fraction of G. xylinus. This unknown product may have been produced by transglycosylation activity of Bgl because it was reported that Bgl from G. xylinus hydrolyzes β-oligosaccharides with retention of the anomeric configuration, and that it also has transglycosylation capability.14 So, the additional product is probably pNP-β-D-gentiobioside judging from the mobility and the gentiobiose-forming ability of G. xylinus described above.

Further analysis of the fraction showing high pNPG1-hydrolyzing activity was done using other p-nitrophenyl-β-D-glycosides (pNP-β-D-glycosides) as substrates. As shown in Fig. 2, the time course of hydrolysis of pNPG3 was observed by TLC. The hydrolysis reactions were carried out at 40°C for 0–60 min in 20 mM SAB (pH 5.5) using 100 μg/mL protein fractions with 4 mM pNPG3 as the substrate in 50 μL-reaction volume. The insoluble fraction of G. xylinus produced pNPG2 mainly after 10 min, and the production of pNPG1 was increased after 20–30 min. Then glucose and p-nitrophenol accounted for the large portion of hydrolysates from pNPG3 after 60 min. This pattern of hydrolysis demonstrates that β-glucosidase present in the insoluble fraction of G. xylinus release glucose from non-reducing end of the substrate in an exo-type fashion. This property of β-glucosidase in the insoluble fraction of G. xylinus is similar to that observed in the enzyme purified from the culture supernatant of G. xylinus.13 Analysis of the G. hansenii (an alternative name for G. xylinus) ATCC23769 sequenced genome (GenBank: ADTV00000000) reveals that only the gene encoding β-glucosidase (bgl), which belongs to GH family 3, is located downstream of the cellulose synthesis operon. Our experimental observations supported by the genome sequence analysis confirm that the β-glucosidase activity found in the insoluble fraction of G. xylinus and that purified from the culture supernatant of G. xylinus is from the same enzyme. We did not detect any β-glucosidase activity in the extracellular fraction of G. xylinus by TLC analysis, and it is reported that the β-glucosidase activity in the extracellular fraction and cytosolic fraction are lower than in the insoluble fraction.5 When the cytosolic fraction of A. bogorensis was used for further analysis of the β-glucosidase activity, no hydrolysis of pNPG3 was detected even after 60 min reaction. From these above observations, it was revealed that A. bogorensis has a similar type of β-glucosidase in the cytosolic fraction. However, its activity is different from β-glucosidase contained at the insoluble fraction of G. xylinus in the aspect of hydrolysis properties. While G. xylinus exhibited the β-glucosidase activity against both cellobiosaccharides and pNP-β-D-glycosides, A. bogorensis exhibited the hydrolytic activities against aryl-β-D-glucosides specifically.

To determine if the protein fractions contained any activity for production of gentiobiose, the condensation reaction was performed according to the method of Kawano et al.5 Each protein fraction (10 mg) was incubated with 100 μL of 50wt% glucose in 20 mM SAB (pH 5.5) at 40°C for 24 h, and the reaction products were analyzed by high performance liquid chromatography (HPLC) equipped with TSKgel Amide-80 column (Φ 4.6 × 250 mm, Tosoh Corporation, Tokyo Japan) and equilibrated with 80% acetonitrile. The column temperature was maintained at 80°C, and the flow rate was kept at 0.8 mL/min. A distinct peak detected at 28 min and corresponded to gentiobiose was observed when the insoluble and cytosolic fractions from G. xylinus were used in the reaction (Fig. 3(b)). When the insoluble fraction of G. xylinus was used, peaks were also detected at 19.5 min and 24 min corresponding to laminaribiose and sorphore, respectively, but these amounts were vanishingly low as compared with gentiobiose. Under similar reaction conditions, peaks corresponding to gentiobiose and other β-disaccharides were not detected when protein fractions from A. bogorensis were used (Fig. 3(c)).

![Fig. 2. Pattern of hydrolysis of pNPG3 by protein fractions containing β-glucosidase activity.](image-url)

(a) Detection of sugar spots, and (b) detection of p-nitrophenol spots. The insoluble fraction was used for G. xylinus, and the cytosolic fraction was used for A. bogorensis. 0, 10, 20, 30 and 60 indicate incubation time in minutes.
peaks detected at around 26 min was detected in both *G. xylinus* and *A. bogorensis* but it has not been identified. The high β-glucosidase activity present in the insoluble fraction of *G. xylinus* probably catalyzes the condensation reaction for production of gentiobiose, while the high β-glucosidase activity present in the cytosolic fraction of *A. bogorensis* does not catalyze this condensation reaction. So, although the cytosolic fraction of *A. bogorensis* exhibits high hydrolytic activity toward pNPG1 (Fig. 1), it does not have the ability to produce gentiobiose.

In a previous study, it was reported that the addition of gentiobiose to culture medium of *G. xylinus* enhanced the activity of CMCase related to cellulose biosynthesis, and gentiobiose was predicted to be produced by β-glucosidase. While the *bgl* gene is found at the region downstream of the cellulose synthesis operon and adjacent to *cesD* in *G. xylinus*, other cellulose-producing bacteria that do not produce as much cellulose, including *A. bogorensis* do not have the β-glucosidase gene downstream of the cellulose synthesis operon. We confirmed that both *G. xylinus* and *A. bogorensis* produced enzymes that hydrolyze pNPG1, but the localization and the enzymatic properties were markedly different. Unlike the enzyme identified as β-glucosidase present in the insoluble fraction of *G. xylinus*, the enzyme observed in the cytosolic fraction of *A. bogorensis* hydrolyzes aryl-β-D-glucosides specifically and it does not have the ability to produce gentiobiose during incubation with cellulose. The lack of ability to produce gentiobiose in *A. bogorensis* may be one of factors for the low productivity of cellulose as a result of no enhancement of the endo-β-1,4-glucanase gene expression.

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**REFERENCES**