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Characterization of cyanobacterial glycogen isolated from the wild type and from a mutant lacking of branching enzyme

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This paper is dedicated to Professor Derek Horton on the occasion of his 70th birthday

Abstract

Cyanobacteria produce glycogen as their primary form of carbohydrate storage. The genomic DNA sequence of *Synechocystis* sp. PCC6803 indicates that this strain encodes one glycogen-branching enzyme (GBE) and two isoforms of glycogen synthase (GS). To confirm the putative GBE and to demonstrate the presence of only one GBE gene, we generated a mutant lacking the putative GBE gene, *sll*0158, by replacing it with a kanamycin resistance gene through homologous recombination. GBE in *sll*0158$^-$ mutant was eliminated; the mutant strain produced less glucan, equivalent to 48% of that produced by the wild type. In contrast to the wild-type strain that had 74% of the glucan being water-soluble, the mutant had only 14% of the glucan water-soluble. Molecular structures of glucans produced by the mutant and the wild type were characterized by using high-performance size-exclusion and anion-exchange chromatography. The glycogen produced by the wild type displayed a molecular mass of 6.6×10^7 daltons (degree of polymerization (DP) 40700) and 10% branch linkages, and the α-D-glucan produced by the mutant displayed a molecular mass of $4.7–5.6 \times 10^3$ daltons (DP 29–35) with slight branch linkages. The results indicated that *sll*0158 was the major functional GBE gene in *Synechocystis* sp. PCC6803.

Keywords: *Synechocystis* sp. PCC6803; Homologous recombination; Glycogen branching enzyme (GBE); Glucan; HPSEC; HPAEC

1. Introduction

Glycogen is a major reserve carbohydrate in both prokaryotes and eukaryotes. It is an α-D-(1→4)-glucan polymer containing about 10% α-(1→6)-branch linkages.[1] Three major enzymes, ADP-glucose pyrophosphorylase, glycogen synthase, and branching enzyme, are involved in bacterial glycogen biosynthesis.[2–4] Glycogen branching enzyme (GBE; (1→4)-α-D-glucan: (1→4)-α-D-glucan 6-α-D-(1→4)-α-D-glucano]-transferase) plays a key role in determining glycogen structure by forming branch linkages. A similar type of BE also is responsible for the branch-structure of starch in plants.

Cyanobacteria and other bacteria are known to produce glycogen as their carbohydrate reserve.[5–7] Genes encoding BE have been cloned from several bacterial species, and their activities have been examined.[8–12] Bacteria generally possess one branching enzyme,[4] whereas plants possess at least two isoforms with different specificities.[13,14] Analysis of the genomic DNA of *Synechocystis* sp. PCC6803 on the basis of sequence similarity with other organisms indicated that this cyanobacterium had one gene encoding a glycogen-branching enzyme.[15] However, this putative GBE gene has not been proven to encode a functional GBE, nor is it certain that no other GBE genes exist in the genome.

It is of additional interest to determine whether a GBE-deficient bacterial mutant could accumulate any

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linear, unbranched glucan material. Studies using iodine staining have shown that GBE-deficient mutants of *Escherichia coli* and yeast accumulate amylose-like glucan.[16,17] It is expected that in GBE-deficient mutants, glycogen synthase (GS) can elongate linear glucan to an unknown length. A reduced efficiency in glucan biosynthesis is expected because of limited non-reducing ends of the linear glucan, compared with branched molecules, that are available for elongation by GS. A GBE-deficient mutant of cyanobacteria can be used to investigate what chain-lengths of linear glucans are synthesized by GS alone.

To determine whether *sll*0158 encodes a GBE and the only functional one, we generated a BE-deficient mutant in *Synechocystis* sp. PCC6803. The yield and structure of the glucan produced by the mutant were studied and compared with that produced by the wild type. Because most linear α-(1→4)-glucans are known not to be soluble in water, it is necessary to develop a method to extract insoluble glucan material from cell debris.

2. Results

Disruption of the sll*0158 gene putatively encoding a GBE in* Synechocystis *sp. PCC6803.*—A mutant (M3) lacking a putative GBE gene, *sll*0158, was generated by transforming a wild-type strain of *Synechocystis* sp. PCC6803 with a plasmid pSHK0158 (Fig. 1). The transformation resulted in a replacement of *sll*0158 with a gene conferring resistance to kanamycin (Km^R)

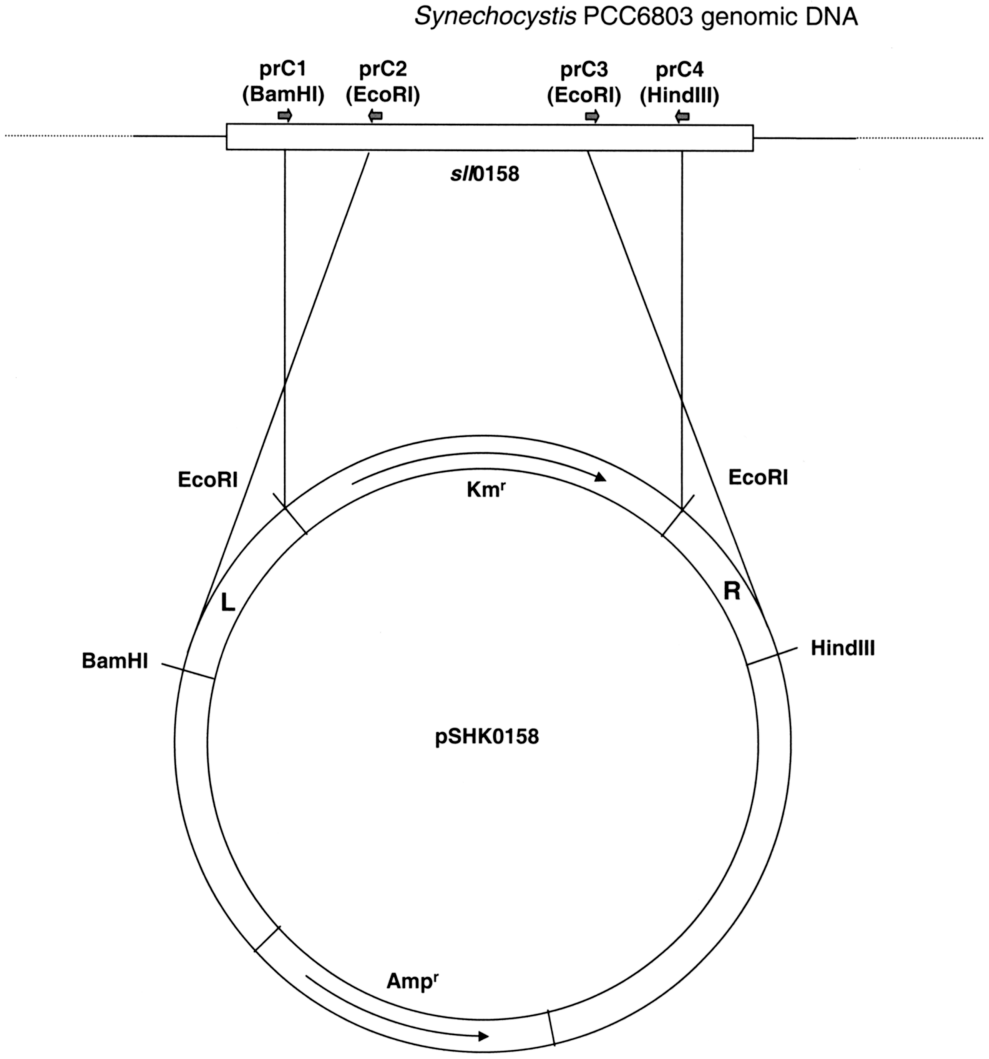

Fig. 1. Construction of a vector for insertional disruption of the *sll*0158. Two homologous DNA sequences were amplified using prC1/prC2 (for left fragment, L) and prC3/prC4 (for right fragment, R), respectively, by PCR. The left homologous DNA fragment was restricted with *Bam*HI and *Eco*RI and the right fragment with *Eco*RI and *Hind*III. Restricted fragments and the Km^R gene were sequentially inserted into pBluescript KS. The resulting vector was used for transformation of wild-type *Synechocystis* sp. PCC6803.

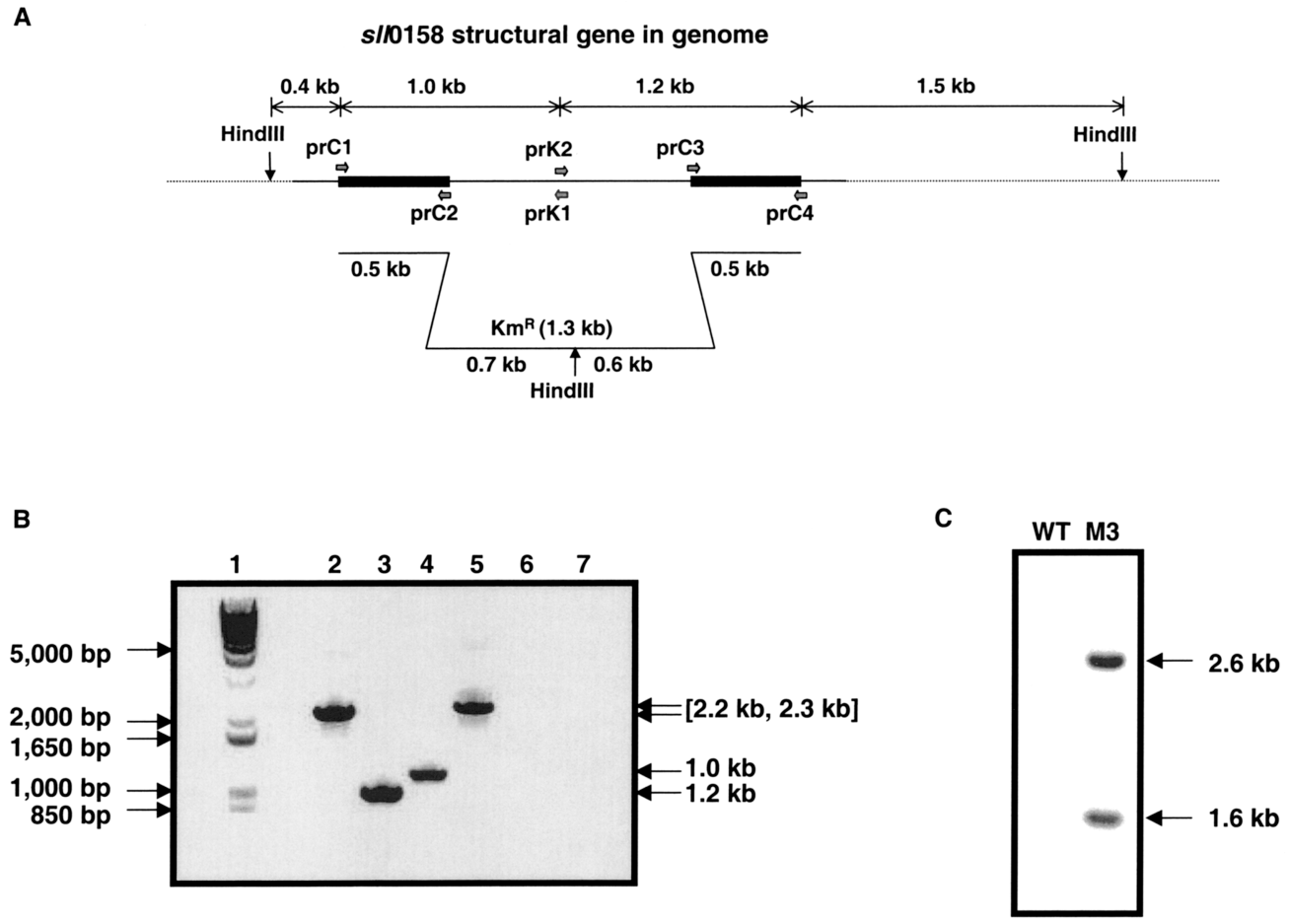

Fig. 2. (A) Gene structure of *sll*0158 in *Synechocystis* sp. PCC6803 genomic DNA. *Hind*III restriction sites are indicated, along with the Km^R gene used for targeted replacement of *sll*0158 by homologous recombination. (B) PCR analysis of the *sll*0158 gene using genomic DNA from wild-type and *sll*0158$^-$ strains as templates. The amplified bands in lanes 2 (WT) and 5 (M3) show similar sizes, because by using prC1 and prC4 primers, the amplified bands for the intact *sll*0158 gene (2.2 kb) and for the inserted Km^R gene (2.3 kb) are almost the same size. When primers of PrC1/PrK1 and PrK2/PrC4 were used, 1.0 and 1.2 kb bands showed on lanes 3 and 4 (WT), respectively, and there were no bands on lanes 6 and 7 (M3). These results showed that the *sll*0158 gene has been replaced with the Km^R gene. Lane 1 is the size marker (1 kb plus DNA ladder, Life Technologies). (C) Southern blot analysis of the mutant and wild-type *Synechocystis* sp. PCC6803. Genomic DNA (5 μg) was digested with *Hind*III and probed with the *Eco*RI-fragment of the Km^R gene.

via homologous recombination. PCR analysis was performed to detect insertion of the Km^R gene cassette into the targeted sequences and to verify complete elimination of wild-type copies of the targeted gene. The structure of *sll*0158 is shown in Fig. 2A. Primers, prC1 and prC4, were used to amplify both the mutagenized and intact *sll*0158 genes, generating the corresponding PCR products (2.3 and 2.2 kb for M3 and WT, respectively). The size difference between PCR products of the mutant and the wild type was less than 100 bp, which was too similar to identify with confidence. Thus, new primers (prK1 and prK2) were designed in the middle of the *sll*0158 gene and utilized to amplify the wild-type gene. The expected sizes of the PCR-amplified fragments were identified in WT (Fig. 2B), but no detectable bands were found in M3, demonstrating that the wild-type *sll*0158 gene was no longer present in M3. Southern hybridization analysis confirmed that the restriction fragment sizes of the mutagenized *sll*0158 genome region corresponded exactly to those expected for the inserted Km^R gene (Fig. 2C). The results confirmed that the targeted *sll*0158 gene was disrupted and completely segregated in the M3 strain. This M3 strain was used as comparison for studying the function of the enzyme encoded by the *sll*0158 gene.

The growth rate of M3 did not appear to be significantly affected by the insertional mutation, compared with that of the wild-type strain (data not shown). Defective structural genes encoding glycogen biosynthetic enzymes also had no effect on the growth of bacteria and yeast.[18,19]

Insertional inactivation of sll*0158 eliminates GBE activity*.—Glycogen-branching enzyme (GBE) activity was assayed over a 1-h time course (Fig. 3) by measuring the amount of inorganic phosphate (P_i) released during a phosphorylase *a* stimulation assay.[20,21] Following a lag of approximately 5 min, the amount of released P_i increased substantially over time for the WT strain. The rate of P_i release from 5 to 60 min was fairly linear for the WT strain (1.5 mM min^{-1} mg^{-1} protein), but no branching activity was detected for M3. This analysis confirmed that the insertional inactivation of the *sll*0158 gene diminished the total cellular GBE activity to an undetectable level.

Glucan extracted from the sll*0158*$^-$ *mutant (M3) is primarily linear with slight branching*.—To examine the effect of the disruption of *sll*0158 on glycogen biosynthesis, we quantified the α-D-glucan produced in M3 and analyzed its structure in comparison with that of glycogen produced in WT. The yield of α-D-glucan produced by M3 was about 48% of that by WT (Table 1), but the structure was very different. The soluble fraction extracted from M3 contained only a small amount of α-D-glucan (2.3 mg g^{-1} cell mass, 14% of total glucan). The majority (86%) of the total α-D-glucan (14 mg g^{-1} cell mass) was found in a cold water-insoluble form present with the cell debris. In contrast, 74% of the glycogen (25.3 mg g^{-1} cell mass) in WT was in a cold water-soluble form (Table 1). High-performance size-exclusion chromatograms (HPSEC) showed that the relative molecular mass (M_r) of the M3 glucan was much smaller than that of the WT glucan (Fig. 4). The M_r of the soluble and insoluble glucan isolated from WT (6.6×10^7 daltons, degree of polymerization (DP) 40700) were identical (Table 2). The M_r of the soluble and insoluble glucans isolated from M3 was 4.7×10^3 (DP $\sim$29) and 5.6×10^3 daltons (DP $\sim$35), respectively.

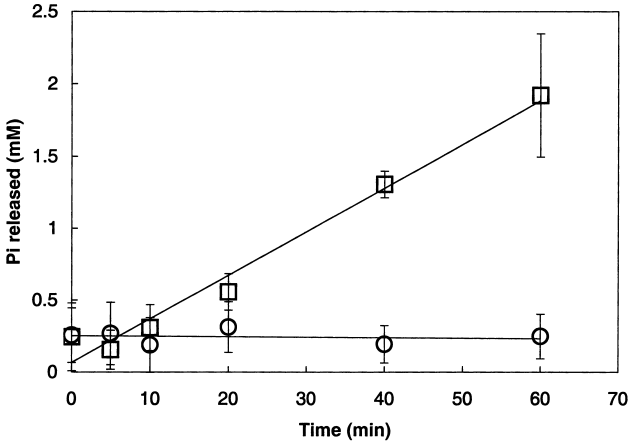

Fig. 3. Time course analysis of glycogen branching enzyme activities from WT (□) and M3 (○), as measured by the phosphorylase *a* stimulation assay.

Table 1
Effect of *sll*0158 deletion on accumulation of glycogen

Source of glucan	Glucan yield (mg g^{-1} wet cell mass)	
	M3	WT
Water-soluble fraction (A)	2.3 ± 0.2 (14%)	25.3 ± 1.1 (74%)
Water-insoluble fraction (B)	14.0 ± 0.5 (86%)	8.8 ± 0.8 (26%)
Total amount (A)+(B)	16.3	34.1
Glucan yield	48%	100%

The isolated glucans were treated with amyloglucosidase and converted to glucose. Total content of α-D-glucan from each source was determined using a glucose diagnosis kit.

The molecular mass profiles of the soluble and insoluble glucans of M3 analyzed by using high-performance anion-exchange chromatography (HPAEC) are shown in Fig. 5A and B, respectively. The HPAEC chromatograms were in agreement with the range of the molecular mass of both fractions of M3 glucans analyzed by using HPSEC. The peak molecular masses of both soluble and insoluble M3 glucans were ca. DP 19. Histograms of quantitative molecular mass distribution of the insoluble M3 glucans with and without isoamylase debranching are shown in Fig. 6A. The results showed increases in smaller molecules (DP 2–16) and decreases in large molecules (DP 17–44) after the isoamylase debranching reaction, which suggested that M3 glucan consisted of some short branched chains. The very large λ_{max} (632 nm) of iodine complex of insoluble M3 glucans (Table 2) also suggested that insoluble M3 glucan had amylose-like structures; some molecules had molecular mass larger than DP 100 as revealed by the HPSEC chromatogram (Fig. 4).

Debranched glycogen of the WT (Fig. 6B) showed a unimodal branch chain-length distribution with the peak chain length of DP 6, typical of the structure of cyanobacterial glycogen. The insoluble fraction of WT-glycogen consisted of longer branch-chains than did the soluble fraction of WT-glycogen (Fig. 6B), which agreed with an increase in λ_{max} from 512 nm for the soluble glucan to 518 nm for the insoluble glucan (Table 2).

3. Discussion

Glycogen branching enzyme (GBE) has a dual function of hydrolyzing an α-(1→4)-linkage and synthesizing an α-(1→6)-linkage. On the basis of the genomic sequence of *Synechocystis* sp. PCC6803, this strain is predicted to encode one GBE as is typical for bacteria. By generating a mutant strain lacking GBE, we demon-

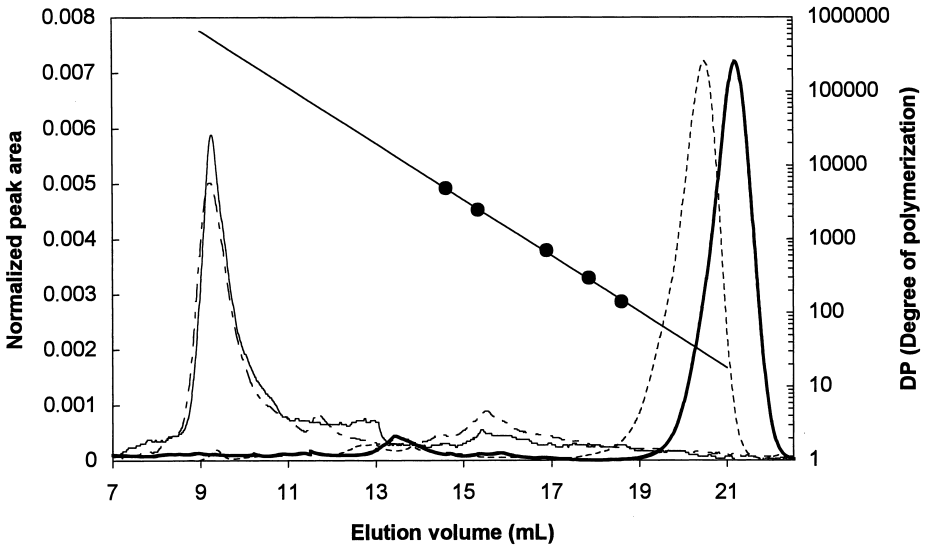

Fig. 4. High-performance size-exclusion chromatograms of the glucans isolated from WT and M3 strains. Peak areas of soluble (▬) and insoluble (----) M3 glucans, and soluble (—) and insoluble (–·–) WT glycogens were normalized. The calibration curve was constructed by using five pullulan standards with molecular mass of 78.8×10^4, 40.4×10^4, 11.2×10^4, 4.73×10^4, and 2.28×10^4 daltons in the order of the data point (●) from the lowest elution volume.

Table 2
Effect of *sll*0158 deletion on glycogen structure

Source of glucan	Molecular mass (M_r[a])		λ_{max}[b]	
	M3	WT	M3	WT
	Dalton (Da)		nm	
Water-soluble fraction	4.7×10^3 (DP 29)	6.6×10^7 (DP 40 700)	546	512
Water-insoluble fraction	5.6×10^3 (DP 35)	6.6×10^7 (DP 40 700)	632	518

[a] Relative molecular mass was calculated from a standard curve of pullulan standards, which was based on the elution time from the HPSEC system.

[b] Glucan–iodine complex was scanned from 400 to 700 nm. Maximum wavelength (λ_{max}) was determined from the absorption spectrum.

strated that the *sll*0158 gene encoded a functional GBE that was responsible for the highly branched structure of glycogen.

Results showed that after the putative GBE gene was disrupted, the cellular GBE activity was diminished. It was confirmed that M3, without GBE activity, produced a reduced amount of primarily linear glucan, equivalent to 48% of that produced in the WT strain. By staining *E. coli* cells with iodine, Romeo et al. suggested that *E. coli* strain lacking GBE synthesized unbranched glucan.[16] Guan et al. reported that glucans produced by an *E. coli* mutant (*glgB*$^-$), missing GBE, had an average molecular weight of DP 42 and a glucan yield of more than 50% of that produced by cells transformed with maize BE.[22] GBE-deficient mutants of *Saccharomyces cerevisiae*, a unicellular eukaryote, have been reported to accumulate approximately 20–60% of the glycogen produced by the wild type.[17]

As a result of lacking GBE activity, once glucan chains are elongated by GS to a sufficient length, the linear chains form double helical crystallites and precipitate out from the solution. The precipitation of the glucan chains hinders further elongation of the chains by GS. As a result, majority of glucans produced by the M3 strain were found precipitated with cell debris and could not be extracted by cold water. The M3 glucans were primarily linear and had average molecular mass of about DP 35, which was 10^4 times smaller than the WT glycogen. The average molecular mass, DP 35, falls in the average folding chain-length of amylose double helices (DP 31).[23] Molecular mass distribution of insoluble M3 glucan varied from DP 15 to 102, as revealed by HPSEC. The λ_{max} of amylose–iodine complex increases linearly with the chain length of amylose and reaches a break point at DP ~ 100.[24] Molecules of DP ~ 100 found in the insoluble M3 glucan were attributed to the large λ_{max} of 632 nm. The soluble M3 glucan had

molecular mass between DP 3 and 40, which were smaller than that of the insoluble glucan. The λ_{max} of the soluble glucan was substantially smaller (546 nm). Moreover, because of the absence of branches, the rate of glucan biosynthesis in M3 was reduced substantially.

From the analysis of the GBE activity using the phosphorylase *a* stimulation assay, we found no detectable branching activity in M3. However, some evidence for branch structures was detected, through HPAEC analysis following isoamylase debranching. Isoamylase treatments of M3 glucan resulted in an increase in short chains and a decrease in long chains in comparison with the untreated M3 glucans. These results suggested that there were some branched molecules, and that branch linkages were hydrolyzed by isoamylase treatments to release short chains. Because BE has highly conserved domains for substrate binding and catalytic sites,[25] the inability to detect another conserved sequence corresponding to a putative BE gene in the genomic sequence suggests there is unlikely to be another GBE isoform. During disruption of *sll*0158 by homologous recombination, four conserved regions that are considered very important for BE activity have been replaced by the antibiotic resistance marker,[25,26] as indicated by the PCR and Southern blot analysis results. Thus, it is highly unlikely that any functional GBE activity could arise from a partial transcript of the *sll*0158 gene. At this time, it is not clear what the source of apparent residual BE activity might be.

In the HPAEC analysis of debranched WT glycogen, there were detectable differences in the branch chain-length distribution between the water-soluble and water-insoluble fractions. The insoluble fraction of WT glycogen showed a larger proportion of longer chains (DP ≥ 14) than the soluble fraction, suggesting that water solubility of glucan is a result of the branch chain-length of the glycogen. Long branch chains of glycogen tended to crystallize became insoluble, and precipitated with the cell debris. The insoluble glycogen could be extracted by using DMSO as the solvent.

Our results showed that the *sll*0158$^-$ mutant, deficient in GBE, had a retarded glucan-synthesizing rate.

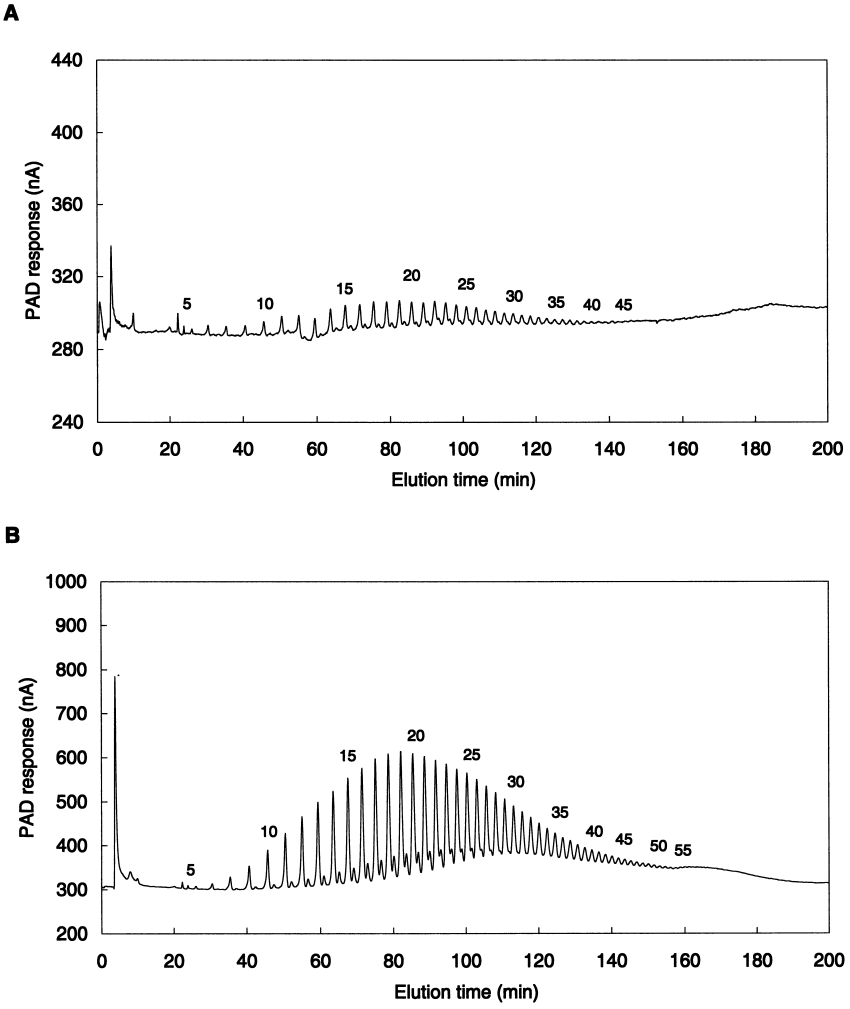

Fig. 5. (A) Molecular mass distribution of the water-soluble fraction of M-3 glucan; (B) the water-insoluble fraction of M3 glucan.

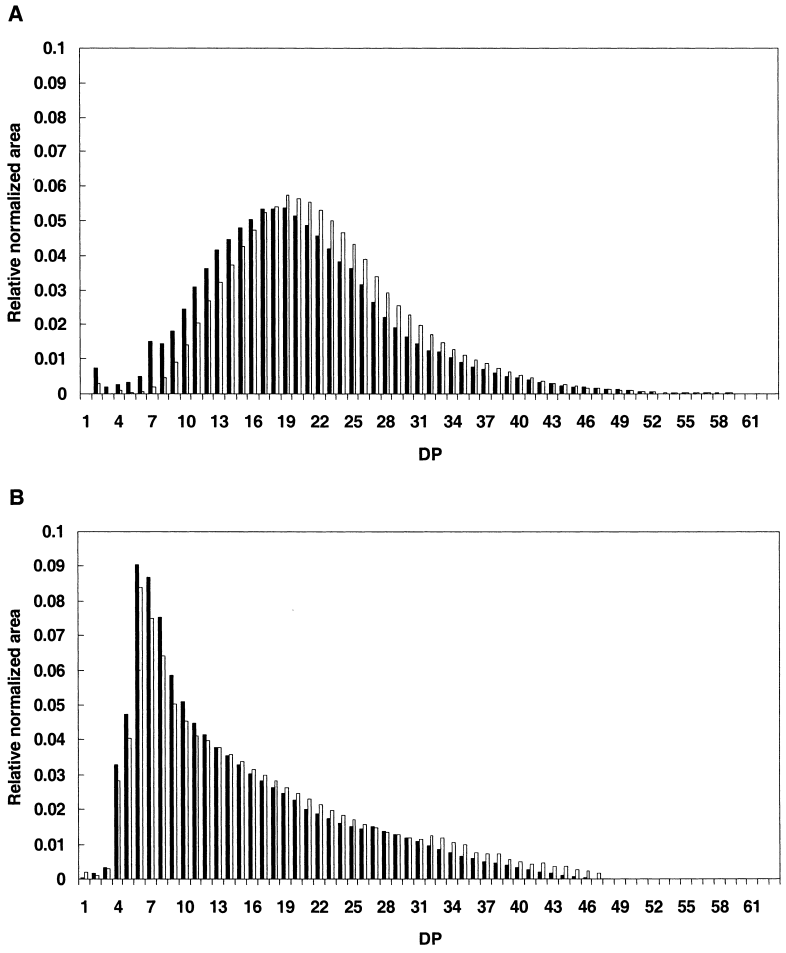

Fig. 6. (A) Histograms of molecular mass distributions of water-insoluble M3 glucans before (□) and after (■) isoamylase treatment (48 h, 40 °C); (B) water-insoluble (□) and water-soluble WT glucans (■) after isoamylase treatment. The peak area was calculated and normalized from the corresponding HPAEC chromatograms.

Most glucans accumulated at an insoluble form with substantially reduced branch linkages.

4. Experimental

Materials.—Chemicals for BG-11 medium were purchased from Fisher Scientific Co. (Pittsburgh, PA). Molecular biology reagents were obtained from Promega Biotech (Madison, WI), and most other chemicals and antibiotics were from Sigma Chemical Co. (St. Louis, MO).

Strains and growth conditions.—*Synechocystis* PCC6803 strains were grown at 25 °C under continuous fluorescent light (40 μE m^2 s^{-1}) in BG-11 media supplemented with 5 mM glucose.[27] For initial growth of the mutant strain, the medium contained 50 μg mL^{-1} kanamycin. For glycogen isolation, cells were grown in a BG-11 medium for 7–8 days ($A_{730} = 1.6–1.8$) before being transferred to a nitrogen-limiting BG-11 medium for another 2–3 days of growth. The nitrogen-limiting BG-11 medium contains less than 10% of the normal sodium nitrate (83.4 mg mL^{-1}) as the only nitrogen source. Cell pellets harvested by centrifugation at $6000 \times g$ for 15 min were washed with sterile deionized water prior to transfer of the cells to the nitrogen-limiting medium. Cultures were started at an initial cell density of 1.0×10^6 cells mL^{-1} for determination of the cell growth rate.

DNA isolation and mutagenesis of the sll*0158 gene in* Synechocystis *sp. PCC6803.*—Genomic DNA of *Synechocystis* sp. PCC6803 was isolated following the procedure of Ausubel et al. with some modifications.[28] A brief scheme for constructing the knock-out plasmid, pSHK0158, is shown in Fig. 1. To replace the putative BE gene, *sll*0158, with a kanamycin resistance (Km^R) gene by a double crossover homologous recombination, we used two PCR-amplified DNA fragments from the coding regions close to the 5′ and 3′ ends of the *sll*0158 gene. Two sets of primers, prC1 (forward, 5′-AATCTTCACCAG/GATCCCTTTGA-3′)/prC2 (reverse, 5′-GATAAAG/AATTCCCACACCATGTT-3′)

and prC3 (forward, 5′-GGCAAAAG/AATTC-CAACGTCAG-3′)/prC4 (reverse, 5′-AAAGACCA/AGCTTCTGTCCATT-3′), were used for PCR amplification. Primers were designed to contain *Bam*HI/*Eco*RI and *Eco*RI/*Hind*III at the 5′/3′ ends of the amplified DNA fragments. The resulting two amplified DNA fragments were subcloned sequentially into a pBluescript KS vector, and the Km^R ($\sim$1.3 kb) gene from pUC4K (Pharmacia) was inserted between them using the *Eco*RI restriction site. Mutant strain (M3) was obtained by transforming *Synechocystis* sp. PCC6803 with the final construct, pSHK0158, and selecting for kanamycin resistance.[29] After several rounds of segregation by single colony selection, insertion of the Km^R gene cassette into the targeted sequence was confirmed in the transformants by PCR and Southern blot analyses. Growth of the wild type and mutant strains was monitored by measuring the absorbance of the cell cultures at 730 nm.

PCR and southern blot analysis of sll*0158 mutant strain.*—Insertion of the Km^R gene cassette into the targeted sequence was confirmed by PCR analysis. The oligonucleotides, prC1 and prC4, which were designed to amplify DNA fragments used for homologous recombination, were used to obtain full-length intact (WT) and Km^R-disrupted (M3) structural genes. Oligonucleotides, prK1 (reverse, 5′-ATAAAGTCTTCGGGACTGCCAA -3′) and prK2 (forward, 5′-TTGGCAGTCCCGAAGACTTTAT-3′) from the middle part of sll0158 replaced with the Km^R gene, were used to confirm the complete absence of the wild-type sll0158 gene in M3. Primer pairs, prC1/ prK1, and prK2/prC4, were used to amplify the left and right regions of sll0158, respectively, in the wild type and M3 strains. For Southern blot analysis, genomic DNA from wild-type and M3 strains was isolated, purified, digested with HindIII, separated in 0.8% agarose gels, and blotted onto nitrocellulose membrane (Hybond-C, Amersham). The DNA probe (a 1,282 bp EcoRI fragment of the Km^R gene) was labeled with [α-^{32}P]-dCTP, and Southern hybridization was performed by standard techniques.[30]

Determination of glycogen-branching enzyme (GBE) activity.—GBE activity was quantified using the phosphorylase *a* stimulation assay.[20,21] The reaction mixture contained 20 μL of 1 mg mL^{-1} rabbit-liver phosphorylase *a* (equivalent to 0.04 units), 1 mM AMP, 100 mM sodium citrate (pH 7.0), 45 mM α-D-glucose 1-phosphate, and 80 μL of cell-free extract (equivalent to 20 μg of total soluble protein) in total volume of 200 μL. The reaction was initiated at 30 °C by adding α-D-glucose 1-phosphate. At different time intervals, 30-μL samples were boiled for 10 min to quench the enzymatic reaction. The resulting sample solutions (30 μL each) were diluted with deionized water, and the P_i released from the reaction was determined as described by Baykov et al.[31] using a Microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) to measure the absorbance at 630 nm in 200 μL of the mixture from each assay. Boiled cell extracts were used as controls at the same reaction condition. Protein concentrations were determined using a Protein Assay Kit (Bio-Rad). Bovine serum albumin (BSA) was used as the standard.

α-D-Glucan extraction from M3 deficient in GBE.—Wild-type and mutant cell cultures (1 L; grown for 2 days in N-limited BG-11 medium as described above) were harvested by centrifugation at $6000 \times g$ for 15 min. The cell pellets frozen with liquid nitrogen were stored at -20 °C until used. The cells were ground and broken in liquid nitrogen using a mortar and pestle, and soluble glucans were extracted by suspending the broken cells in 10 mL of deionized water and washed two more times with an equal volume of water. After centrifugation ($10000 \times g$ for 15 min), the supernatants containing soluble glucan were removed and boiled in a water bath for 15 min. The cell debris residues (1.2 g, wet basis) were dispersed in 4 mL of dimethyl sulfoxide (DMSO) and heated in a water bath for an hour with stirring to dissolve insoluble glucan. The supernatants containing the insoluble glucan, obtained after centrifugation ($8000 \times g$, 15 min), were precipitated with 3 vol. of EtOH at 4 °C overnight. The glucan pellets, recovered by another centrifugation ($8000 \times g$ for 15 min), were again dispersed in DMSO and recovered as described above. Residual DMSO in the glucan precipitate was removed with two more EtOH washes (0.5 mL each), and the glucan precipitate was dried in the air. The dried glucan was dissolved in 10 mL of boiling water and heated in a boiling water bath for 30 min, and the resulting solutions containing insoluble glucan were used for further study.

Yield and structure of α-D-glucan and λ_{max} of α-D-glucan–iodine complex.—Yields of α-D-glucans were determined using an enzymatic assay and a phenol–sulfuric acid method. For the enzymatic assay, α-D-glucans were completely hydrolyzed with amyloglucosidase (Sigma Chemical Co.) (5 units mg^{-1} total carbohydrate) at 55 °C for 2 h, and the amount of released glucose was quantified using a glucose diagnosis kit (Sigma Chemical Co.). Total carbohydrate content was also determined using a phenol–sulfuric acid method.[32] Iodine staining of the glucan dispersion (0.5 mg mL^{-1}) was prepared in a solution containing 0.01% I_2 and 0.1% KI. Absorbance of the iodine complex was scanned from 700 to 400 nm to determine the wavelength of maximum absorbance (λ_{max}). The relative molecular mass (M_r) of the glucan was determined by using an HPSEC system.[33] Pullulan standards P-82 (Shodex, Japan) were used to calibrate the analytical columns. The branch chain-length distribution of glucans was determined by using an HPAEC system (Dionex-300, Sunnyvale, CA) equipped with an amy-

loglucosidase reactor and a pulsed amperometric detector (PAD). The operating condition of the HPAEC analysis was established previously.[34]

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