

Molecular cloning and disruption of a novel gene encoding UDP-glucose:tetrahydrobiopterin α -glucosyltransferase in the cyanobacterium *Synechococcus* sp. PCC 7942

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Abstract The gene encoding UDP-glucose:tetrahydrobiopterin α -glucosyltransferase (BGluT) was cloned from the genomic DNA of *Synechococcus* sp. PCC 7942. The encoded protein consisting of 359 amino acid residues was verified *in vitro* and *in vivo* to be responsible for the synthesis of tetrahydrobiopterin (BH4)-glucoside produced in the organism. The BGluT gene is the first cloned in pteridine glycosyltransferases and also a novel one cloned so far in UDP-glycosyltransferases. The mutant cells disrupted in the BGluT gene produced only aglycosidic BH4 at a level of 8.3% of the BH4-glucoside in wild type cells and exhibited half of the wild type growth in normal photoautotrophic conditions. These results suggest that the glucosylation of BH4 is required for the maintenance of the high cellular concentration of the compound, thereby supporting the normal growth of *Synechococcus* sp. PCC 7942. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Tetrahydrobiopterin-glucoside; Glucosyltransferase; Pteridine glycosyltransferase; Gene disruption overexpression; Cyanobacterium; *Synechocystis* sp. PCC 6803

1. Introduction

Unconjugated pteridine compounds are ubiquitous in nature as cofactors and pigments [1]. Tetrahydrobiopterin (BH4) is best known as a cofactor for aromatic amino acid hydroxylation and nitric oxide synthesis in higher animals [2]. On the other hand, pteridine glycosides, which have various kinds of sugars attached to the side chain at C-6 of the pterin ring such as biopterin, 6-hydroxymethylpterin, and neopterin, are abundant in some microbes [3–12]. They have been found exclusively in cyanobacteria [3–9], the anaerobic photosynthetic bacteria *Chlorobium limicola* [10] and *Chlorobium tepidum* [11], and the chemoautotrophic archaeobacterium *Sulfolobus solfataricus* [12]. However, the cellular function of pteridine glycosides remained unknown, although earlier studies postulated a role in electron transport in photosynthesis [13,14].

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Abbreviations: biopterin, 6-(L-erythro-1',2'-dihydroxypropyl)-pterin; BH4, (6R)-5,6,7,8-tetrahydro-L-biopterin; cyanopterin, 6-[1-(4-O-methyl- α -D-glucuronyl)-(1,6)-(β -D-galactosyloxy)methyl]pterin; BGluT, UDP-glucose:tetrahydrobiopterin α -glucosyltransferase

More recently, protecting against UV damage or trapping the energy for use in photosynthesis was also proposed as a possible function [15,16]. Pteridine glycosides are produced in large quantities, especially in cyanobacteria [3–9]. In *Synechocystis* sp. PCC 6803 the intracellular concentration of cyanopterin is comparable to that of chlorophyll *a*, thereby suggesting some other essential role rather than a cofactor function [8]. Another important property of pteridine glycosides may be that, like the functional pteridine compound BH4, they exist *in vivo* as fully reduced tetrahydro forms, which are easily converted to dihydro and further to oxidized forms. These characteristics together with the glycosidic form may be implicated in the cellular function of the compounds.

From the beginning of our research on the pteridine glycosides, we were interested in the enzyme catalyzing the transfer of sugar to the pterin ring, because it was presumed that the putative function of the compounds is conferred by the sugar moiety. Therefore, the enzymes, named pteridine glycosyltransferases as a novel group of glycosyltransferases [9], were considered important as a useful target for gene disruption study in the relevant organisms. With the aim of gene cloning a pteridine glycosyltransferase was purified from the cyanobacterium *Synechococcus* sp. PCC 7942 [9], in which a BH4-glucoside was identified. The enzyme, named UDP-glucose:BH4 glucosyltransferase (BGluT), catalyzes the synthesis of the BH4-glucoside by transferring glucose from UDP-glucose to BH4. In a subsequent work, the exact chemical structure of the BH4-glucoside was elucidated to be 1-O-(L-erythro-biopterin-2'-yl)- α -glucose by nuclear magnetic resonance spectroscopy, supporting that BGluT belongs to the α -glucosyltransferases [17]. The purified protein provided useful structural information for the subsequent cloning of the gene [9], which was performed in this study. The determined 13 residues of the N-terminal sequence of BGluT shared high homology with the corresponding sequence of a putative protein encoded by slr1166 of the genome-sequenced cyanobacterium *Synechocystis* sp. PCC 6803. Further homology analysis of the putative protein of slr1166 exhibited a region of highly conserved sequence at the C-terminal half for glycosyltransferases. These results predicted that BGluT might have two domain structures, which seems common in a variety of UDP-glycosyltransferases having different acceptor specificities: the variable N-terminal half for pteridine binding and the conserved C-terminal for sugar binding.

In this study, the BGluT gene was cloned from the genomic DNA of *Synechococcus* sp. PCC 7942 and disrupted in the organism in order to investigate the cellular function of BH4-

glucoside. The data on initial characterization of the mutant show that interruption of this gene leads to reduced pteridine production of only the aglycosidic form and retarded growth.

2. Materials and methods

2.1. Growth conditions

Synechococcus sp. PCC 7942 wild type and mutant strains were grown at 30°C in BG-11 medium in batch culture under continuous white light (100–120 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and air bubbling. Growth was measured by the optical density at 730 nm.

2.2. Cloning of the BGLuT gene and inactivation

Degenerate and inverted PCR methods were employed for the cloning of the BGLuT gene from the total genomic DNA of *Synechococcus* sp. PCC 7942. The priming sites of the PCR primers are indicated at the corresponding positions in Fig. 1. Firstly, to clone a partial BGLuT gene two degenerate PCR primer pairs were designed based on a previous report [9]. The forward primers were ATGACNGCN-CAYCGNTTY (F-1) and CGSTTYCTNTTYGTNTC (F-2), which were derived from the determined N-terminal sequence of BGLuT [9]. The reverse primers were CCCRCANGCNAANGCYTC (R-1) and CCRAANGCYTCNACCCA (R-2), which were derived from the consensus amino acid sequences EALACG and WVEAFG, respectively, of the slr1166 in *Synechocystis* sp. PCC 6803. PCR amplification was performed with the outer primer pair (F-1 and R-1) using the genomic DNA as a template, and subsequently with the inner primers (F-2 and R-2) using an aliquot of the first PCR product as a substrate. For inverted PCR cloning [18] two primer pairs were designed from the cloned partial gene sequence of BGLuT. Forward primers were GGGCTTCGTCAGTACCGA (IF-1) and GGCCT-GTTAATGACCCCA (IF-2), located at the 3'-end. Reverse primers were CGTTTCAGCGGCAAAT (IR-1) and GCGAGCAGCT-GACTTG (IR-2), complementary to the nucleotides positioned at the 5'-end. Total genomic DNA was exhaustively digested with *Bam*HI and ligated to obtain circular DNA molecules, which was employed as a template for PCR with the outer primers (IF-1 and IR-1). The PCR product was subsequently used as a template for the second PCR performed with the inner primers (IF-2 and IR-2).

PCR amplifications were performed with Pfu polymerase (Promega) in 1 \times reaction buffer, 200 μM dNTPs, 0.5 μM each of primer pairs, and templates, under the following conditions: 4 min at 95°C, followed by 30 cycles of 95°C for 1 min, 57–65°C depending on the primer pairs for 1 min, and 72°C for 1 min, and a final DNA polymerization at 72°C for 7 min. PCR product was cloned into the pGEM-T vector (Promega) and sequenced.

To construct a knockout vector the 2.2 kb kanamycin resistance gene was digested from pHP45Q-Km with *Bam*HI and ligated into the corresponding site located in the middle of the BGLuT gene, which had previously been cloned in pGEM T-Easy vector. *Synechococcus* cells were transformed following the protocol described in [19]. Positive colonies were restreaked to single ones with at least five serial transfers to obtain full segregation of the mutation.

2.3. Expression and purification of recombinant protein

The complete open reading frame (ORF) sequence of the BGLuT gene was amplified from the genomic DNA using a primer pair, the forward primer (CATATGACTGCCACCGTTTCT) tailed with a *Nde*I sequence (underlined) and the reverse one (GAATTCCTA-GAAGCCTCGGGCTC) with a *Bam*HI sequence (underlined). The PCR product was cloned into the pGEM-T vector and then subsequently subcloned as a *Nde*I-*Bam*HI restriction fragment into the pET-28a expression vector (Novagen).

Escherichia coli strain BL21(DE3) was transformed with the pET-28a plasmid harboring the PCR product. The transformed cells were induced to overexpress the cloned gene with 0.3 mM IPTG and continued to grow overnight. The harvested cells were washed with lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole), resuspended in the same buffer, and disrupted by sonication. The crude extract obtained after centrifugation was applied to a column of Ni-NTA gel (Qiagen) and purified according to the product manual. The recombinant BGLuT protein was eluted with 50 mM imidazole in the lysis buffer. The purified protein was dialyzed against 10 mM PIPES (pH 7.5) and assayed.

2.4. Assays of BGLuT, pteridine compounds, and chlorophyll *a*

The assay of BGLuT activity was performed following the method previously described [9]. The reaction mixture consisted of 50 mM sodium phosphate, pH 7.5, 10 mM MnCl_2 , 0.2% ascorbic acid, 50 μM BH₄, 500 μM UDP-glucose, and enzyme solution. The reaction was carried out for 30 min at 37°C. The reaction mixture was oxidized by addition of an equal volume of acidic iodine solution (2% KI/1% I₂ in 1 N HCl) for 1 h in the dark. After centrifugation, the supernatant was mixed with one tenth volume of 5% ascorbic acid to reduce the excess iodine and subjected to HPLC (see below). Intracellular pteridine compound in *Synechococcus* cells was analyzed by HPLC as described previously [8]. Cells harvested by centrifugation were suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and oxidized as described above. Biopterin-glucoside was quantified as an equivalent to biopterin. Pteridine compounds were purchased from Dr. B. Schirck's Lab (Jona, Switzerland). Biopterin-glucoside of *Synechococcus* sp. PCC 7942 was from the previous isolation [17].

HPLC was performed with a Kontron Model 430 system. Samples of 20 μl were applied on an Inertsil ODS-3 C18 column (5 μm , 150 \times 2.3 mm, GL Science, Japan) equilibrated with water for quantitative analysis or 10 mM potassium phosphate buffer (pH 6.0) for qualitative analysis. Pteridine compounds were eluted isocratically at a flow rate of 1.2 ml/min and monitored at 350/450 nm (excitation/emission) by using a fluorescence detector (HP Model 1046A).

Chlorophyll *a* concentration was determined following the method of [20].

2.5. SDS-PAGE, Southern blot, and Western blot analyses

For Southern blot analysis, 10 μg of total genomic DNA was digested with *Eco*RI and electrophoresed on 1% agarose gel. The gel was transferred to nitrocellulose membrane (Hybond-N⁺, Amersham) and hybridized with the random-primed probe of the BGLuT gene. Hybridization was performed at 65°C for 18 h and the membrane was washed with 0.1 \times SSC containing 0.1% SDS at 65°C twice for 15 min and then exposed to X-ray film. SDS-PAGE was performed with a 12.5% polyacrylamide gel under reducing conditions. For Western blot analysis, electrophoretic transfer to nitrocellulose membrane was carried out in Tris-glycine buffer, pH 8.3. For immunoblot of BGLuT, anti-mouse BGLuT antiserum was used at a dilution of 1:2000. The polyclonal mouse antiserum was prepared against purified recombinant BGLuT. A second antibody conjugated with alkaline phosphatase was used for visualization.

3. Results

3.1. Molecular cloning and sequence analysis

Both the determined N-terminal amino acid sequence and the conserved amino acid sequences of glycosyltransferases derived from the putative structural information of BGLuT [9] were successfully utilized for cloning a partial BGLuT gene from the total genomic DNA of *Synechococcus* sp. PCC 7942. The nested degenerate PCR amplified a DNA product of 799 bp from the genomic DNA (the sequence is indicated in Fig. 1). The deduced amino acid sequence of the clone was highly homologous to that of the putative ORF slr1166 in *Synechocystis* sp. PCC 6803, supporting that the cloned DNA corresponded to a partial gene sequence of BGLuT. In order to clone the missing 5'- and 3'-regions of the BGLuT gene the inverted PCR method was employed. The inner PCR primers amplified a unique DNA band of approx. 3 kb from the first PCR product (data not shown). DNA sequencing of approx. 400 nucleotides at both ends of the clone revealed the complete ORF sequence of BGLuT gene including some neighboring sequences at both ends as shown in Fig. 1 (GenBank accession number AF331846). The ORF sequence consisting of 1080 nucleotides would encode a protein of 359 amino acid residues. The N-terminal sequence of the deduced protein coincides exactly with the determined amino acid sequence of the purified BGLuT [9]. The deduced

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TTGGCAATCTCTTCTTGGGTGAAAAGCTGGGCATGATCCAATGGGTCGGGGTCAGCCTTAGCCTGGTCA 69
GTATTTTGCTGATCAGTCAGCGGGATTGGTTGACAGCCCAGGCTGGAAAACCCGACTCCAGCCCGCTG 138
TTATCGATCCGGCTCTACCTGAAGTCCCCGATCGCTCAGCCCAGTAGCAGCATCCCCAGCTTTTCTTAA 207
                                     F-1       F-2
                                     M T A H R F L F V S T
CCGCGATCGCGCCAGCATCCCTCGGAGGCGTTTCTATGACTGCCACCGTTTCTGTTTGTATCAACAC 276
P V G P L G S G R G G G V E L T L P N L A K A 34
CCGTCGGCCCTTGGGGTCGGGGCAGGGGGCGGTGTTGAACTGACCTGCCCAATTTAGCCAAGGCTC 345
L T Q R G H Q V S V L A P A G S V L P D L P L 57
TGACCCAGCGAGGGCACCAAGTCAGCGTGTCTCGCGCCGCTGGATCGGTGTGCCCCGATTGCGCGTGG 414
                                     IR-2
E T V P G T W Q S T A Q S H G R A T P A E I P 80
AAACGGTGCCTGGGACTTGGCAAAGCACGGCTCAAAGTCACGGGCGGCCACTCTGTGCTGAGATTCTCTG 483
IR-1
A E S V L A R L W D R A H Q Q Q A D F D L I L 103
CTGAGAGTGTTTAGCCCGGCTCTGGGATCGAGCTCACCAGCAGCAAGCCGACTTTGACCTGATCCTCA 552
N F A Y D W L P L Y L T P F F K T P V A H L I 126
ACTTCGCCTACGACTGGCTACCGCTATATCTGACGCCGTTTTTCAAGACGCTGTGCGCCATTGTATCA 621
S M G S L S E V M D Q A I A T S L D R Y P G S 149
GCATGGGATCCCTATCTGAGGTGATGGATCAGGCGATCGCCACAAGCCTCGATCGCTATCCCGGAGCA 690
BamHI
I A V H S L A Q A A T F P F G D R C L C I G N 172
TCGCTGTCCACAGTCTGGCTCAGGCAGCAACCTTTCCCTTTGGCGATCGCTGCCTTTGTATCGGCAATG 759
A L D L A A Y G F N P E P E P V L G W V G R I 195
CCCTTGACCTAGCAGCCTATGGGTTTAAACCAGAGCCAGAGCCGGTCTTGGGCTGGGTAGGACGGATTG 828
A P E K G L E D A I Q A A Q Q A G L P L R V W 218
CACCAGAAAAGGGCTAGAGATGCCATTCAAGCCGCTCAACAGGCTGGCTCCCGTTGCGGGTCTGGG 897
G A L T E P D Y W Q R L Q Q Q F G D R A V S Y 241
GTGCCCTGACTGAACCCGACTATTGGCAACGACTCCAACAGCAGTTTGGCGATCGGGCCGTCAGCTATC 966
Q G F V S T D E L Q R G L G R C Q G L L M T P 264
AGGGCTTCGTAGTACCGATGAGTTGCAACGTGGCTTGGGCGCTGCCAAGGCTGTATGACCCCAA 1035
IF-1       R-2                                     R-1       IF-2
K W V E A F G N V A I E A L A C G L P V I A Y 287
AATGGGTAGAAGCCTTTGGCAACGTCGCCATTGAAGCCCTCGCCTGTGGGCTACCACTGATAGCCTATG 1104
A R G G P L E I I E Q G K S G W L V E P D Q Q 310
CCCGCGGCGGACCTTGGAAATTATTGAGCAGGGCAAGAGTGGCTGGCTGGTAGAACCCGATCAACAGG 1173
A A L V N A I G Q L S S L D R A Y C R A Q A E 333
CTGCTCTCGTCAACGCAATTGGGCAACTGTCTAGCCTCGATCGCGCTTACTGTGAGCCCAAGCCGAAG 1242
A R F S L A A M G Q R L E A W L L P L L S R A 356
CACGGTTTTCTTGGCTGCGATGGGTCAACGTCTAGAAGCGTGGCTACTCCCCCTCTTGTCCCGAGCCC 1311
R G F * 359
GAGGCTTCTAGTTGCGATCGCATTTTCGGCATCGGGCAGTAGCGCCAGCAATAGCCAGCCGTGACGGGG 1380
ACTTCAATCACTTCGCTGAGGGCAGCGCCCAAGGCAACGCATT 1423

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Fig. 1. The nucleotide sequence of the BGluT gene and the deduced amino acid sequence. Both sequences are numbered on the right side. The open reading frame consisting of 1080 nucleotides starts from position 243 and ends at 1322. The amino acid sequences used for the design of degenerate PCR primers (F-1,2 and R-1,2) are boxed and the partial gene sequence (255–1054) cloned by the second degenerate PCR is indicated in italics. Primer sequences for inverted PCR (IR-1,2 and IF-1,2) are underlined by arrows pointing in the direction of polymerization. The determined 13 residues of the N-terminal sequence are shown in bold italics. The enzyme site of *Bam*HI for insertion of the kanamycin resistance gene is indicated.

molecular mass was 38 751 Da, which is highly similar to that of the purified BGluT on SDS-PAGE [9]. These results strongly support that the cloned gene encodes BGluT of *Synechococcus* sp. PCC 7942.

To confirm that the cloned gene encodes BGluT by its enzymatic activity, the ORF sequence amplified by PCR from the genomic DNA was cloned in T7-based vector

pET-28a in correct reading frames and then heterologously overexpressed in *E. coli*. The soluble recombinant protein harboring a N-terminal His-tag sequence was purified to homogeneity on a Ni-NTA column (data not shown). The purified recombinant protein assayed with BH4 and UDP-glucose as substrates showed the genuine activity of BGluT to catalyze the formation of BH4-glucoside, which was iden-

tified as the oxidized bioppterin-glucoside by HPLC (data not shown). Therefore, it became evident that the cloned gene encodes BGLuT.

To our knowledge, the BGLuT gene is the first one cloned so far in pteridine glycosyltransferases. Homology search against the translated nucleotide database exhibited many proteins but mostly similar to the C-terminal half of BGLuT. A few of them, showing overall sequence similarities, were from those microorganisms producing pteridine glycosides. As expected, the putative protein of 354 amino acid residues encoded by srl1166 in *Synechocystis* sp. PCC 6803 was the most significant one having a sequence identity of 45%. The others were from *S. solfataricus* (GenBank accession number CAC23841) and *C. tepidum* (unannotated ORF), having sequence identities of around 20%. The homologous proteins also consisted of around 350 amino acid residues, increasing the probability that they are pteridine glycosyltransferases.

The BGLuT protein was confirmed to consist of two domain structures by computational sequence analyses. Most of the proteins or putative proteins found in the homology search were UDP-glycosyltransferases sharing homologies only at the C-terminal regions. Pfam search [21] also revealed that BGLuT has a conserved amino acid sequence of the glycosyltransferases group 1 at the C-terminal region spanning the residues 183–335 (Fig. 1). These findings indicate that the BGLuT gene is a novel one cloned so far in UDP-glycosyltransferases and support the previous prediction [9] that BGLuT has a two domain structure of a variable N-terminal region and a conserved C-terminal for sugar binding. In further support of these structural characteristics, the putative protein folding recognition of BGLuT searched in 3D-PSSM Web Server [22] exhibited the highest score with the X-ray structures of *E. coli* UDP-*N*-acetylglucosamine 2-epimerase [23], *E. coli* membrane-associated glycosyltransferase [24], and T4 phage β -glucosyltransferase [25], which have well defined two domain structures.

3.2. Inactivation of the BGLuT gene

To investigate the effect of BGLuT gene disruption in *Syn-*

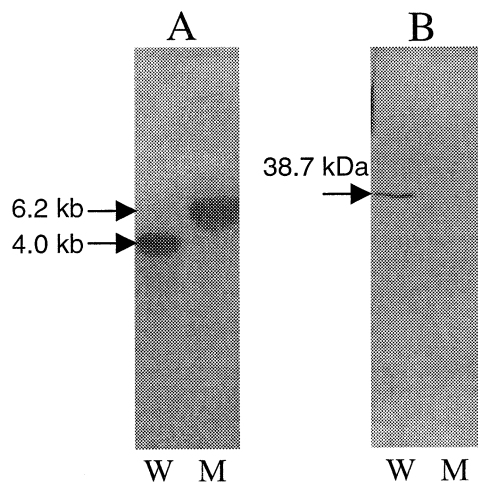


Fig. 2. Inactivation of the BGLuT gene in *Synechococcus* sp. PCC 7942. A: Southern blot analysis of *Eco*RI-digested genomic DNAs of wild type (W) and knockout mutant (M). B: Western blot analysis of total proteins of wild type (W) and the mutant (M) separated by SDS-PAGE on a 12.5% polyacrylamide gel in denaturing conditions.

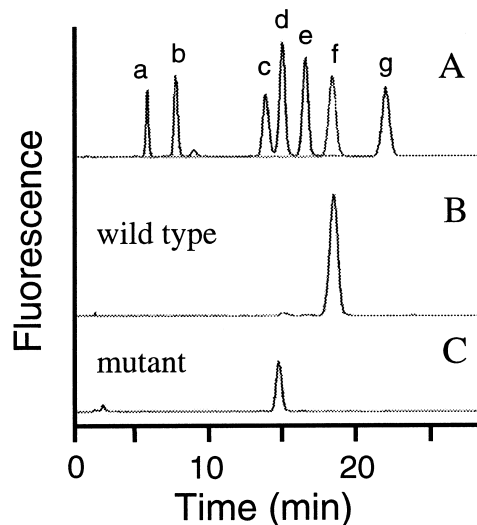


Fig. 3. HPLC analysis of intracellular pteridine compounds in the wild type and mutant cells. A: Standard pteridines: a, neopterin; b, cyanopterin; c, pterin; d, bioppterin; e, 6-hydroxymethylpterin; f, bioppterin-glucoside isolated from *Synechococcus* 7942; g, dictyopterine. B: The intracellular bioppterin-glucoside detected in the wild type. C: The intracellular bioppterin in the mutant. The pteridine compounds in the harvested cells were oxidized by acidic iodine solution and chromatographed on a C18 column isocratically with 10 mM sodium phosphate, pH 6.0, at a flow rate of 1.2 ml/min. Fluorescence detection was at 350/450 nm (em/ex).

echococcus sp. PCC 7942 knockout mutants were created by using a knockout vector containing the 2.2 kb kanamycin resistance gene cassette in the *Bam*HI site of the BGLuT gene (see Fig. 1). Dozens of transformants, obtained after several rounds of segregation, were analyzed by PCR to show that they all harbor only a disrupted BGLuT gene. Among them a transformant was chosen for further analysis of both the genotype and phenotype. Complete disruption of the BGLuT gene in the transformant was confirmed by genomic Southern (Fig. 2A) and Western blot analyses (Fig. 2B). Absolutely no wild type copy of the BGLuT gene or BGLuT protein was detected in the mutant cells. Finally, HPLC analysis of the mutant cells detected no bioppterin-glucoside but only bioppterin (Fig. 3), which, furthermore, was in much reduced amount (see below). The peak eluting at the corresponding position of bioppterin was confirmed by coinjection with the standard bioppterin. This result of blockage in the last step of BH4-glucoside synthesis is a clear demonstration in vivo that the BGLuT gene is completely disrupted in the mutant cells.

3.3. Phenotype analysis of the knockout mutant

The mutant was analyzed for growth and pteridine production in normal photoautotrophic conditions (Fig. 4). Although the mutant was viable, the growth measured at 730 nm was approximately two-fold slower compared to the wild type (Fig. 4A). Their chlorophyll *a* levels also showed the same pattern. However, the pteridine concentration was much lower in the mutant, yielding approx. 8.3% of wild type in a normalized value to chlorophyll *a* concentration (Fig. 4B). Fig. 4B is a plot of molar ratio against chlorophyll *a* concentration, manifesting their linear correlation; the approximate value of wild type was 1.2, while that of the mutant was 0.1. We analyzed the cell medium for extracellular pteridines but

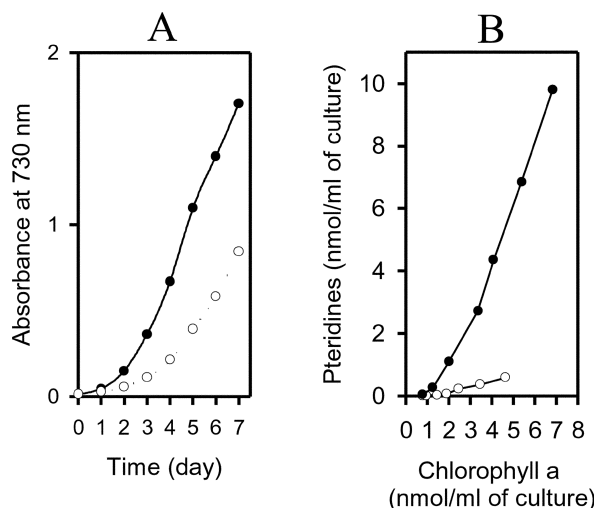


Fig. 4. Comparative analyses of cell growth and pteridine production in both wild type (closed circles) and the mutant (open circles). A: Cell growth measured at 730 nm. B: Intracellular pteridine concentration plotted against chlorophyll *a* concentration. The approximate linear regression values for the slopes were 1.2 and 0.1 in the wild type and the mutant, respectively.

detected no measurable amounts of any kinds of pteridine compounds. It indicates that the low biopterin level in the mutant cells did not result from its excretion to the medium but probably from reduced synthesis. The disruption of the BGluT gene could have polar effects on the expression of neighboring genes, thereby reducing the growth rate. However, analysis of the downstream sequence of the BGluT gene does not show the presence of any potential gene within the determined sequence, which excludes the possibility of any polar effects on cell growth. Therefore, these results suggest that glucosylation of BH4 affects the cellular concentration of the compound, which in turn affects cellular growth.

4. Discussion

In this study the BGluT gene was cloned and inactivated in *Synechococcus* sp. PCC 7942 for the purpose of investigating the putative function of BH4-glucoside or its sugar moiety. The functionality of the cloned BGluT gene was proved both in vitro and in vivo. The complete absence of BH4-glucoside in the mutant *Synechococcus* cells (Fig. 4) is a clear demonstration that the encoded protein BGluT catalyzes the synthesis of the tetrahydro form of the compound, the chemical structure of which was determined to be 1-*O*-(L-erythro-biopterin-2'-yl)- α -glucose [17], as a retaining group of glucosyltransferase. Therefore, the full name of BGluT is revised here as UDP-glucose:tetrahydrobiopterin α -glucosyltransferase.

BGluT is the first gene cloned so far in pteridine glucosyltransferases, which should be present in all those bacteria producing pteridine glycosides. This result would contribute much to the identification of other pteridine glucosyltransferases and furthermore the microbial distribution of pteridine glycosides. The functional identification of the most putative one encoded by slr1166 of *Synechocystis* sp. PCC 6803, which is currently being done, will be valuable for completing the

unfinished biosynthetic pathway of cyanopterins [26]. Other putative ones of the archaeobacterium *S. solfataricus* (CAC23841) and the anaerobic photosynthetic bacterium *C. tepidum* also deserve functional identification, because subsequent homology searches using their deduced sequences exhibited putative ORFs in *Bradyrhizobium japonicum* (AJ003064), *Mesorhizobium loti*, and several archaeobacteria such as *Aeropyrum pernix* (BAA80177) and *Methanothermobacter thermoautotrophicus* (AAB84679), which are so far unknown for the presence of pteridine glycosides.

The results obtained with the knockout mutant strongly suggest that the glucosylation of BH4 is required for the maintenance of the high cellular concentration of the compound, thereby supporting normal growth. The putative function of the sugar moiety in BH4-glucoside was expected to be either a crucial role in the cellular function of the compound such as transforming the bioactivity of the compound or a secondary role implementing the function. If it were the former case, the mutant producing only aglycosidic BH4 would not have survived in the normal growth condition. Therefore, the result that the mutant is alive supports the latter more plausible case. This secondary role of sugar may allow the variations of the sugar residues found hitherto in pteridine glycosides [5–12]. The fact that the mutant contains a smaller amount of BH4 suggests that the secondary role of glucosylation might be increasing the solubility or stability of BH4, thereby contributing to the high intracellular concentration of the compound. This suggestion implies a quantitative role of the pteridine compound, irrespective of the presence of a glucose moiety in it, for normal growth. As suggested in the previous study on *Synechocystis* sp. PCC 6803, the extremely high intracellular concentration of BH4-glucoside (Fig. 4B) supports a role not relevant to a cofactor function. A property of pteridine glycosides possibly related to the quantitative role might be their fully reduced tetrahydro states in vivo.

Although our present result does not suggest a decisive role of BH4-glucoside itself, it is obvious that the retarded growth of the mutant clearly reflects some defective physiological process. Therefore, future in-depth characterization of the mutant will provide valuable information on the cellular function of BH4-glucoside in *Synechococcus* sp. PCC 7942 including other pteridine glycosides.

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