

# A Cyanobacterial Gene, *sqdX*, Required for Biosynthesis of the Sulfolipid Sulfoquinovosyldiacylglycerol

SINAN GÜLER,<sup>†</sup> BERND ESSIGMANN, AND CHRISTOPH BENNING\*

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824-1319

Received 9 July 1999/Accepted 21 October 1999

**The sulfolipid sulfoquinovosyldiacylglycerol is present in the photosynthetic membranes of plants and many photosynthetic bacteria. A novel gene, *sqdX*, essential for sulfolipid biosynthesis in the cyanobacterium *Synechococcus* sp. strain PCC7942 is proposed to encode the cyanobacterial sulfolipid synthase catalyzing the last reaction of the pathway.**

One of the most ubiquitous sulfur-containing bioorganic compounds is the sulfolipid sulfoquinovosyldiacylglycerol (11). It is a typical constituent of photosynthetic membranes in plants and many photosynthetic bacteria (2, 13). Four genes, *sqdA*, *sqdB*, *sqdC*, and *sqdD*, encoding enzymes of the sulfolipid biosynthetic pathway, as determined by genetic analysis, were first isolated from the purple bacterium *Rhodobacter sphaeroides* (3, 4, 17). Orthologs of the *sqdB* gene were subsequently cloned from the cyanobacterium *Synechococcus* sp. strain PCC7942 (hereafter referred to as *Synechococcus*) and *Arabidopsis thaliana* (8, 10). A mutant of *R. sphaeroides* inactivated in *sqdD* with sequence similarity to glycosyltransferase genes accumulates UDP-sulfoquinovose (17). Based on this observation, it has been proposed that the *sqdD* gene product transfers sulfoquinovose from UDP-sulfoquinovose onto a suitable acceptor, presumably diacylglycerol, during the final step of sulfolipid biosynthesis. The recent completion of the genomic sequence of the cyanobacterium *Synechocystis* sp. strain PCC6803 (hereafter referred to as *Synechocystis*) (14) provided the opportunity to search for cyanobacterial orthologs of the of *R. sphaeroides* *sqd* genes. Although a putative *sqdB* ortholog was present, none of the other *sqd* sequences of *R. sphaeroides* matched sequences in the cyanobacterial genome.

**Identification of *sqdX*.** Genes encoding enzymes in the same biosynthetic pathway are often organized in operons, and novel cyanobacterial genes involved in specific biosynthetic pathways, e.g. vitamin E biosynthesis (18), have been successfully isolated based on this assumption. Employing a similar approach, the entire insert of the previously isolated plasmid pSYB (10) carrying the *sqdB* gene of *Synechococcus* was sequenced (GenBank accession no. AF155063), leading to the identification of a new open reading frame (ORF) directly downstream of *sqdB* (Fig. 1A). This ORF encodes a putative protein of 377 amino acids with no sequence similarity to any of the described *sqd* gene products of *R. sphaeroides* (3, 4, 17). Unlike the preceding *sqdB* ORF which starts with GTG, the second ORF begins with ATG 15 bp downstream of the *sqdB* gene. An ideal ribosome binding site is not present, but an AAAG sequence 14 bp upstream of the ATG may serve as such. We designate this ORF *sqdX*. Analysis of the deduced amino acid sequence of *sqdX* employing Pfam (protein families

database of alignments) (1) revealed a glycosyltransferase group 1 domain between the residues 228 and 347. Members of this family transfer activated sugars, for example UDP-, ADP-, GDP-, or CMP-linked sugars, to a variety of substrates, including fructose-6-phosphate and glycogen (6). Furthermore, based on the analysis with TopPred (19) and TMPred (12), the protein contains two hydrophobic domains, indicating that the *sqdX* gene product might be a transmembrane protein.

**Disruption of *sqdX* in *Synechococcus* leads to sulfolipid deficiency.** To test whether *sqdX* is indeed essential for sulfolipid biosynthesis, a gene replacement approach was employed (Fig. 1A). A 1.5-kb *SalI/BamHI* fragment of the plasmid pSYB (10) containing the entire *sqdX* gene and the carboxy-terminal coding region of the *sqdB* gene was subcloned into pBluescript II SK<sup>+</sup> (Stratagene). This subclone was digested with *PstI* to remove a 0.9-kb fragment internal to the *sqdX* gene. The deleted fragment was replaced with a 1.2-kb *PstI* fragment carrying the aminoglycoside 3'-phosphotransferase gene of pUC4K (Pharmacia) conferring kanamycin resistance. The resulting plasmid pDL2 (Fig. 1A) was used to transform wild-type *Synechococcus* as previously described (10). Repeated restreaking of transformants on agar-solidified (1.5% wt/vol) BG-11 medium (7) with 25 µg of kanamycin ml<sup>-1</sup> resulted in the complete loss of all wild-type genome copies. This was confirmed by DNA-DNA hybridization of *HindIII*-digested genomic DNA probed with a fragment covering *sqdX* (Fig. 1A). Under highly stringent hybridization conditions, the wild type showed a diagnostic fragment approximately 6.0 kb in length which was entirely replaced by fragments of approximately 5.1 and 1.2 kb (Fig. 1B) in the mutant strain designated 7942Δ*sqdX*. This strain completely lacked sulfolipid based on labeling experiments with [<sup>35</sup>S]sulfate (Fig. 2) which were performed as previously described (10). Taken together, these results strongly suggest that *sqdX* encodes an enzyme essential for sulfolipid biosynthesis in *Synechococcus*.

***Synechococcus* and *Synechocystis* differ in the organization of *sqd* genes.** A search for the *sqdX* gene in the recently completed genome sequence of *Synechocystis* (14) identified the putative ORF slr0384 (Cyanobase [www.kazusa.or.jp/cyanobase/index.html]) encoding a protein with 72% identity over the entire length compared to the *sqdX* gene product of *Synechococcus*—starting with the first in-frame ATG (position 2386042 of the genome sequence). However, the *sqdB* gene of *Synechocystis* (slr1020) as annotated in the Cyanobase with an amino acid sequence identity of 44% between the two orthologs was located approximately 1.8 Mb downstream from the putative *sqdX* gene. Unlike in *Synechococcus*, this arrangement excludes the possibility that the two *sqd* genes of *Syn-*

\* Corresponding author. Mailing address: Department of Biochemistry, Michigan State University, East Lansing, MI 48824-1319. Phone: (517) 355-1609. Fax: (517) 353-9334. E-mail: benning@pilot.msu.edu.

<sup>†</sup> Present address: Universitätsklinikum Charité, Institut für Pharmakologie und Toxikologie, 10117 Berlin, Germany.

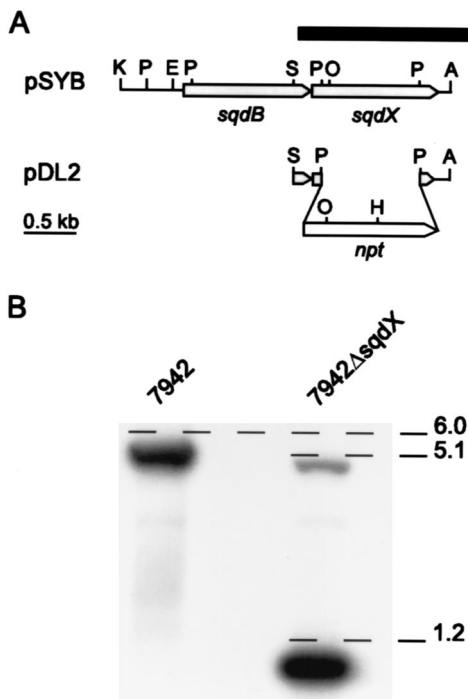


FIG. 1. Insertional inactivation of *sqdX*. (A) Restriction and ORF map of the plasmid pSYB and the inactivation plasmid pDL2. The black bar indicates the location of the probe used for hybridization, and the open arrow indicates the neomycin phosphotransferase cassette. Restriction sites: A, *Bam*HI; E, *Spe*I; H, *Hind*III; K, *Kpn*I; O, *Xho*I; P, *Pst*I; S, *Sal*I. (B) Autoradiograph of a Southern blot showing genomic DNA from *Synechococcus* wild type and 7942 $\Delta$ *sqdX* mutant. The DNA was cleaved with *Hind*III and probed with the fragment indicated in A. The approximate size of the three diagnostic fragments is indicated in kb.

*echocystis* are cotranscribed. To obtain proof that the putative *sqdX* gene of *Synechocystis* indeed encodes an enzyme essential for sulfolipid biosynthesis, we attempted to inactivate ORF slr0384 in *Synechocystis*. By using primers 5'-CGCGGATCCA TGCGTGTGCCCCTGTTT-3' and 5'-CCCAAGCTTCTAA GCCGTAACGGAGCGT-3', the putative *sqdX* ORF of *Synechocystis* was PCR cloned into *Hind*III/*Bam*HI-digested pUC18 plasmid. A *Sal*I fragment derived from pUC4K carrying the aminoglycoside 3'-phosphotransferase gene was inserted into the *Xho*I site of *sqdX* in pUC18. Transformation of *Synechocystis* with this plasmid and selection of *sqdX* inactivation mutants was done under the same conditions as for *Synechococcus* (10). However, despite multiple rounds of restreaking, we were unable to obtain a sulfolipid-deficient mutant strain of *Synechocystis* lacking wild-type genome copies as determined by lipid analysis and DNA-DNA hybridization (data not shown). Either the insertion into the *sqdX* gene affects the expression of another essential gene near to it, or sulfolipid is essential for the growth of *Synechocystis* under the employed conditions.

***Synechocystis sqdX* restores sulfolipid biosynthesis in *Synechococcus* mutant 7942 $\Delta$ *sqdX*.** To confirm that the *sqdX* genes in both cyanobacteria indeed encode functionally homologous proteins, we inserted the *sqdX* ORFs of *Synechococcus* and of *Synechocystis* behind the *tac* promoter in the mobilizable broad-host-range plasmid pRL59EH (5) and transferred the constructs by conjugation into *Synechococcus* mutant 7942 $\Delta$ *sqdX* as described (20). Because it was not clear where the *sqdX* ORF of *Synechocystis* starts, we included sequences upstream of the presumed ATG up to the first in-frame stop

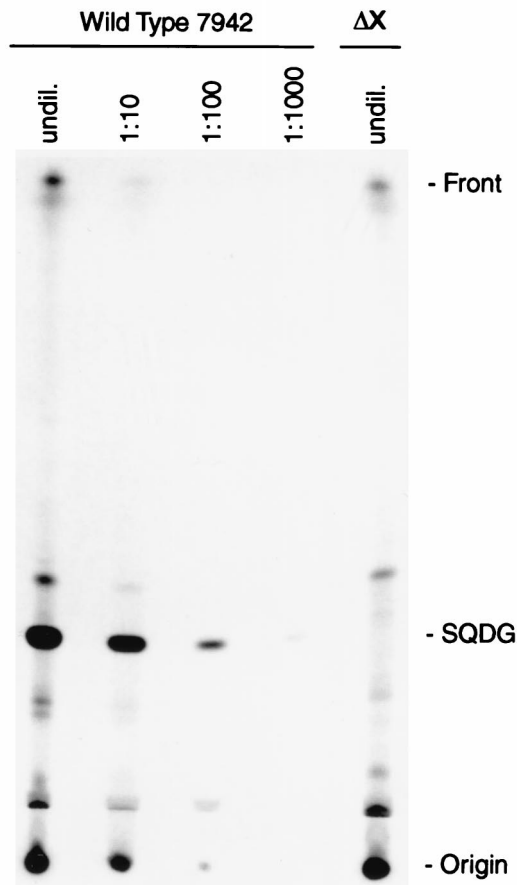


FIG. 2. Separation of  $^{35}$ S-labeled lipids from *Synechococcus* wild type and 7942 $\Delta$ *sqdX* mutant ( $\Delta$ X). Either equal amounts of undiluted lipids (undil.) or serial dilutions of the wild-type extract, as indicated, were spotted. The sulfolipid band is marked SQDG. Compounds were visualized by autoradiography.

codon (positions 2385912 to 2387168 of the genome sequence). The *sqdX* gene of *Synechococcus* was PCR cloned from the plasmid pSYB (Fig. 1) by using the primers 5'-AAGGATCC TGCGCTAAAGTCGCACTC-3' and 5'-ATAAGCTTCGAG CTCAGGCCGCT-3' into the *Hind*III/*Bam*HI sites of pRL59EH. In the same way, *sqdX* of *Synechocystis* was cloned from genomic DNA using primers 5'-CGGGATCCTATTCT AGGTTGGCCAC-3' and 5'-CCCAAGCTTCTAAGCCGC TAACGGAGCGT-3'. Finally, an omega cassette from the plasmid pHP45 $\Omega$  (16) conferring spectinomycin and streptomycin resistance was inserted into the *Hind*III sites of these plasmids to provide a suitable selectable marker. The resulting plasmids containing *sqdX* of *Synechococcus* or *Synechocystis* were designated pSQDX7942 or pSQDX6803, respectively. Exconjugants were selected on BG11 medium containing 25  $\mu$ g of kanamycin ml $^{-1}$ , 10  $\mu$ g of spectinomycin ml $^{-1}$  and 1  $\mu$ g of streptomycin ml $^{-1}$  and were analyzed by DNA-DNA hybridization to confirm the presence of the proper plasmid constructs (data not shown). Both constructs restored the sulfolipid biosynthetic activity in the *Synechococcus* mutant 7942 $\Delta$ *sqdX* (Fig. 3) as shown by thin-layer chromatography lipid analysis (10). Based on the observed genetic complementation, it can be concluded that both cyanobacterial *sqdX* genes encode proteins involved in sulfolipid biosynthesis and that *sqdX* of *Synechocystis* is an ortholog of the respective gene of

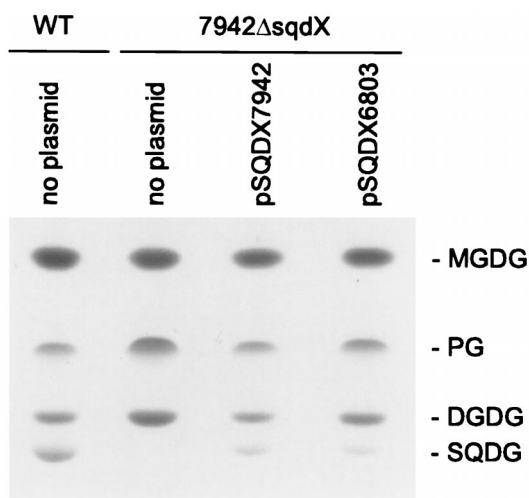


FIG. 3. Genetic complementation of the *Synechococcus* 7942 $\Delta$ sqdX mutant. Lipid extracts from *Synechococcus* wild type (WT) and mutant strain 7942 $\Delta$ sqdX carrying plasmids as indicated were separated by thin-layer chromatography. Lipids were visualized by exposure to iodine vapor. DGDG, diagalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol

*Synechococcus*, despite different gene organization and different susceptibility for inactivation by insertional mutagenesis.

**Evolution of the sulfolipid biosynthetic pathway.** Unlike *sqdB*, no sequences with similarity to *sqdA*, *sqdC*, or *sqdD* of *R. sphaeroides* were detected upon examination of the *Synechocystis* genomic sequence, suggesting that some aspects of the pathway are evolutionarily highly conserved, while others are not. The bacterial *sqdB* (3, 10) and the plant *SQD1* (8) genes encode highly conserved proteins with similarity to sugar-nucleotide-modifying enzymes which are proposed to be involved in the biosynthesis of the UDP-sulfoquinovose headgroup donor for sulfolipid biosynthesis (9, 15). The underlying reaction seems to be unique to sulfolipid biosynthesis, and thus, the capability to synthesize sulfoquinovose evolved only once. However, the apparent lack of conservation of other genes involved in the final assembly of sulfolipid even between photosynthetic bacteria suggests that different glycosyltransferases may have been recruited to catalyze this reaction in different organisms. Our discovery of the putative glycosyltransferase involved in sulfolipid biosynthesis in cyanobacteria was initially based on the assumption that genes encoding enzymes of the same metabolic pathway are often organized in transcriptional units. This strategy worked in the case of *Synechococcus*, but would have failed if *Synechocystis* had been chosen as the model organism. Inactivation of *sqdX* in *Synechococcus* led to complete sulfolipid deficiency which could be restored by introducing the *sqdX* genes of either *Synechococcus* or *Synechocystis* in *trans*. These two results provide sufficient evidence to conclude that the *sqdX* gene product is essential for cyanobacterial sulfolipid biosynthesis, at least in *Synechococcus* and presumably also in *Synechocystis*. Furthermore, the sequence similarity to glycosyltransferases strongly suggests that *sqdX* encodes the sulfolipid synthase catalyzing the transfer of sulfoquinovose from UDP-sulfoquinovose onto a suitable acceptor, presumably diacylglycerol. Accordingly, the two putative membrane spanning domains predict a tight membrane association of this enzyme. The isolation and genetic characteriza-

tion of *sqdX* now provide the means to study the last reaction of sulfolipid biosynthesis in greater detail in cyanobacteria. It remains to be seen whether the *sqdX* gene product may also serve as a model for plant sulfolipid synthases.

This work was supported in part by grant DE-FG02-98ER20305 from the Department of Energy.

We thank Peter Wolk for helpful discussion during the course of this work and his criticism of the manuscript. In addition, we thank Michael Peters-Kottig for her efforts to inactivate *sqdX* in *Synechocystis* and Jamie Hubert for her technical assistance.

#### REFERENCES

- Bateman, A., E. Birney, R. Durbin, S. R. Eddy, R. D. Finn, and E. L. Sonnhammer. 1999. Pfam 3.1: 1313 multiple alignments and profile HMMs match the majority of proteins. *Nucleic Acids Res.* **27**:260–262.
- Benning, C. 1998. Membrane lipids in anoxygenic bacteria, p. 83–101. In P. A. Siegenthaler and N. Murata (ed.), *Lipids in photosynthesis: structure, function and genetics*. Kluwer Academic Publishers, Boston, Mass.
- Benning, C., and C. R. Somerville. 1992. Identification of an operon involved in sulfolipid biosynthesis in *Rhodobacter sphaeroides*. *J. Bacteriol.* **174**:6479–6487.
- Benning, C., and C. R. Somerville. 1992. Isolation and genetic complementation of a sulfolipid-deficient mutant of *Rhodobacter sphaeroides*. *J. Bacteriol.* **174**:2352–2360.
- Black, T. D., and C. P. Wolk. 1994. Analysis of a Het- mutation in *Anabaena* sp. PCC7120 implicates a secondary metabolite in the regulation of heterocyst spacing. *J. Bacteriol.* **176**:2282–2292.
- Campbell, J. A., G. J. Davies, V. Bulone, and B. Henrissat. 1997. A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem. J.* **326**:929–939.
- Castenholz, R. W. 1988. Culturing methods for cyanobacteria. *Methods Enzymol.* **167**:68–93.
- Essigmann, B., S. Güler, R. A. Narang, D. Linke, and C. Benning. 1998. Phosphate availability affects the thylakoid lipid composition and the expression of *SQD1*, a gene required for sulfolipid biosynthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **95**:1950–1955.
- Essigmann, B., B. M. Hespeneide, L. A. Kuhn, and C. Benning. 1999. Prediction of the active-site structure and  $\text{NAD}^+$  binding in *SQD1*, a protein essential for sulfolipid biosynthesis in *Arabidopsis*. *Arch. Biochem. Biophys.* **369**:30–41.
- Güler, S., A. Seeliger, H. Härtel, G. Renger, and C. Benning. 1996. A null mutant of *Synechococcus* sp. PCC7942 deficient in the sulfolipid sulfoquinovosyl diacylglycerol. *J. Biol. Chem.* **271**:7501–7507.
- Heinz, E. 1993. Recent investigations on the biosynthesis of the plant sulfolipid, p. 163–178. In L. J. De Kok (ed.), *Sulfur nutrition and assimilation in higher plants*. SPB Academic Publishing, The Hague, The Netherlands.
- Hofmann, K., and W. Stoffel. 1993. TMbase: a database of membrane spanning proteins segments. *Biol. Chem. Hoppe-Seyler* **374**:166.
- Imhoff, J. F., and U. Bias-Imhoff. 1995. Lipids, quinones and fatty acids of anoxygenic photosynthetic bacteria, p. 179–205. In R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), *Anoxygenic photosynthetic bacteria*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Kaneko, T., S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirasawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, and S. Tabata. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* **3**:109–136.
- Mulichak, A. M., M. J. Theisen, B. Essigmann, C. Benning, and R. M. Garavito. 1999. Crystal structure of *SQD1*, an enzyme involved in the biosynthesis of the plant sulfolipid headgroup donor UDP-sulfoquinovose. *Proc. Natl. Acad. Sci. USA* **96**:13097–13102.
- Prentki, P., and H. M. Krisch. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. *Gene* **29**:303–313.
- Rossak, M., C. Tietje, E. Heinz, and C. Benning. 1995. Accumulation of UDP-sulfoquinovose in a sulfolipid-deficient mutant of *Rhodobacter sphaeroides*. *J. Biol. Chem.* **270**:25792–25797.
- Shintani, D., and D. DellaPenna. 1998. Elevating the vitamin E content of plants through metabolic engineering. *Science* **282**:2098–2100.
- von Heijne, G. 1992. Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J. Mol. Biol.* **225**:487–494.
- Wolk, C. P., A. Vonshak, P. Kehoe, and J. Elhai. 1984. Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing filamentous cyanobacteria. *Proc. Natl. Acad. Sci. USA* **81**:1561–1565.