The sulfolipid 6-sulfo-quinovosyl diacylglycerol is found in the photosynthetic membranes of all plants and most photosynthetic bacteria. Progress toward the elucidation of the pathway for sulfolipid biosynthesis has been slow in the past. However, the recent isolation of three genes of the photosynthetic bacterium Rhodobacter sphaeroides known to be involved in sulfolipid biosynthesis provides promising new opportunities. Two of the genes flank an open reading frame predicted to encode a protein with amino acid sequence similarity to sugar nucleotide-dependent glycosyltransferases. The UDP-sulfoquinovosydiaac glycerol sulfoquinovosyltransferase thought to catalyze the last step of sulfolipid biosynthesis belongs to this group of glycosyltransferases. To test whether this open reading frame encodes the sulfoquinovosyltransferase of R. sphaeroides, it was inactivated by gene replacement avoiding polar mutagenesis. The resulting sulfolipid-deficient mutant defines a new gene, designated sqdD. Mutant cells grown in the presence of [35S]sulfate accumulate a water-soluble 35S-labeled compound. The purified compound was tentatively identified by co-chromatography with standards and enzymatic conversion as UDP-sulfoquinovose, the final precursor of sulfolipid biosynthesis. This result strongly suggests that the inactivation of sqdD causes a metabolic block in the last step of sulfolipid biosynthesis.

The sulfolipid sulfoquinovosydiaacylglycerol is found in all plants and most photosynthetic bacteria (1, 2). In higher plants it is confined to the chloroplast (3). Since the discovery (4) and structural elucidation (5) of sulfoquinovosydiaacylglycerol by Benson and co-workers, different pathways for the biosynthesis of the 6-sulfoquinovosyldiacylglycerol have been suggested (see summary in Ref. 1). Experimental verification of the proposed steps of the pathway is generally lacking, but evidence is accumulating that the immediate precursors from which sulfolipid is assembled are UDP-sulfoquinovose and diacylglycerol. The tentative identification of nucleotide sulfoquinovose in extracts of [35S]sulfate-labeled cells of Chlorella (6) led Benson (5) to suggest that the transfer of the sulfoquinovose moiety from a nucleotide sulfoquinovose onto diacylglycerol may represent the last step of sulfolipid biosynthesis. More recently, it was demonstrated that chemically synthesized UDP-sulfoquinovose could stimulate sulfolipid biosynthesis by spinach chloroplast membranes which were biochemically preloaded with radioactive diacylglycerol (7). Since the effect was specific for UDP-sulfoquinovose as compared to other sulfosugar nucleotides, it was concluded that in spinach chloroplasts a UDP-sulfoquinovosydiaacylglycerol sulfoquinovosyltransferase catalyzes the last step of sulfolipid biosynthesis. Using synthetic UDP-sulfoquinovose as substrate, the biochemical properties of the enzyme and its association with the inner envelope membrane of the chloroplast could be determined (8).

Currently, the only genes known to encode proteins involved in the biosynthesis of sulfoquinovosydiaacylglycerol are sqdA, sqdB, and sqdC isolated from the photosynthetic bacterium Rhodobacter sphaeroides (9, 10). Two of the genes, sqdB and sqdC, were found to flank an open reading frame predicted to encode a protein with an amino acid sequence similar to that of glycogenin (9), a sugar nucleotide-dependent glycosyltransferase, but no mutant affecting this open reading frame was available. Given this sequence similarity and the location of the open reading frame within one of the sulfolipid operons of R. sphaeroides, it is tempting to predict that the open reading frame may encode a protein catalyzing the transfer of sulfoquinovose during final assembly of the sulfolipid in analogy to the reaction in spinach chloroplasts. As a first step to test this hypothesis, we had to specifically inactivate this open reading frame, avoiding polar mutagenesis. This experiment would answer the general question whether the open reading frame represents a novel sqd gene of R. sphaeroides essential for sulfolipid biosynthesis. In addition, we set out to identify UDP-sulfoquinovose in the mutant cells which we expected to accumulate due to a metabolic block in the final step of sulfolipid biosynthesis, if the protein encoded by the open reading frame would be involved in catalyzing this final reaction.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions—Escherichia coli strains used were HB101 (F− Δ(mcr-cmr-mpr) leu2 supE44 araL4 galK2 lacY1 proA2 rpsL20 (Strr) yfy-5 mtl-1 recA13) (11), MM294 (F− endA1 hsdR17 (r− m−) supE44 thi-1 relA1) (12), S17-1 (C600::RP4-2(Tc;Mn)(Km;Tn) thi pro hsdR hsdM Δ recA) (13). R. sphaeroides mutants were derived from wild type strain 2.4.1 (14). The plasmid vectors pCHB500 (9), pBluescript-KS+ (Stratagene), pUC4K (Pharmacia), pSU202 (13), and pCHB160018 (10) were employed for construction of different plasmids as described in the text, and pPK2013 (15) was used as helper plasmid in triparental matings.

R. sphaeroides cell cultures were grown phototrophically in Sistrom's medium (17, 18) as described previously (9). To obtain low sulfate medium, sulfate salts in Sistrom's medium were substituted with chloride salts. E. coli strains were grown in Luria broth. Antibiotics were added at concentrations of 100 μg/ml ampicillin, 50 μg/ml
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kanamycin, or 10 μg/ml tetracycline for E. coli cultures or 25 μg/ml kanamycin for R. sphaeroides cultures.

Construction of the Inactivation Cassette and Plasmid pMR4D—The plasmid pMR1 containing the new inactivation cassette (see Fig. 1) was constructed by ligating a 1.3-kb EcoRI/HindIII neomycin phosphotransferase gene fragment of pUC4K treated to remove sticky ends and redigested with PstI, a 300-bp EcoRI/HindIII fragment of pUC8B500 treated to fill in the 3′-ends and redigested with PstI, which contained the cytochrome c₃ promoter (Pyoc) from Rhodobacter capsulatus (19), and the PstI vector fragment of pUC4K. This procedure ensured the cloning of the promoter fragment in proper orientation at the 3′-end of the neomycin phosphotransferase gene which was confirmed by restriction analysis.

Construction of the plasmid pMR4D used to inactivate the open reading frame flanked by the sqdB and sqdC genes was accomplished in three steps. A 3.6-kb partial PstI/HindIII fragment from pCHB160018 containing the sulfolipid operon from R. sphaeroides was cloned into pBluescript-KSII+ giving rise to plasmid pMR2. This plasmid was cut with EcoRI and ligated with a 1.6-kb EcoRI fragment of pMR1 containing the new inactivation cassette. In the resulting plasmid pMR2D1, a 53-bp fragment of the targeted open reading frame was replaced with the inactivation cassette. In the resulting plasmid pMR2D1, the neomycin phosphotransferase gene was replaced by the inactivation cassette.

Precultures of R. sphaeroides for sugar analysis was not available, different modes of detection were used. An aliquot of 100 μl of the UDP-sulfoquinovose mixture, an excess of alkaline phosphatase (0.6 unit, Sigma) in 10 μl of reaction buffer in 3 μl of water and subjected to further analysis.

Preparation of Chloroplast Membranes and Glycosyltransferase Assay—Approximately 20 g of leaf material of 6-week-old spinach plants were harvested and homogenized in 4°C cold isolation medium (40 mM Tricine/KOH, 300 mM sorbitol, pH 8.0) by four short bursts in a Waring blender. The extracts were kept at 4°C during the whole procedure. Following filtration through two layers of Miracloth, the suspension material was analyzed by thin layer chromatography as described above.

Among the labeled sulfosugars and nucleotides, sulfoquinovose, was prepared according to Bochner and Ames (20). Sulfoquinovose was prepared from synthetic sulfoquinovose 1-phosphate by acid hydrolysis with 1 M aqueous hydrochloric acid for 1 h at 90°C followed by equilibration for 40 min. Since an on-line mass detector for sugar analysis was not available, different modes of detection were used: recording the UV absorption at 254 nm followed in line by radio detection employing a solid-phase flow-through scintillation detector (Bethold LB 507) or manual collection of fractions for calorimetric determination of sugar content and parallel scintillation counting.

High Performance Liquid Chromatography of Synthetic Sulfofuosugars and Nucleotide Standards— Different sulfoquinovose nucleotide standards was used as indicated in the text: solvent A, ethanol/water/acetic acid (20:10:1, by volume); solvent B, isopropanol/dioxane/H₂O/acetic acid (35:10:10:1, by volume). The column was returned to start conditions with a linear gradient over 10 min ending with 100% solvent B.

RESULTS

Inactivation of the Open Reading Frame Flanked by sqdB and sqdC—To test whether the open reading frame located between sqdB and sqdC in the sulfolipid operon of R. sphaeroides represents a new gene involved in sulfolipid biosynthesis, we had to inactivate this putative gene, but allow for the expression of sqdC. From previous experiments it was known that sqdB and sqdC, which are essential for sulfolipid biosynthesis in R. sphaeroides, form a transcriptional unit with sqdC at the 3′-end. To ensure the expression of sqdC, we modified the inactivation cassette from pUC4K by cloning the cytochrome c₃ promoter of R. capsulatus in outward reading orientation behind the 3′-end of the neomycin phosphotransferase gene (Fig. 1). This promoter was successfully used before to express sqdC (10). After deleting a 53-bp EcoRI fragment, this new cassette was inserted into the center of the target open reading frame. The insertional disruption central portion of the operon carried by a plasmid not replicating in R. spha-
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Labeled Water-soluble Compound—The sequence similarity between the protein predicted to be encoded by sqdD and the glycosyltransferase glycogenin suggested that sqdD may encode the sulfooquinovosyl transferase catalyzing the last step of sulfolipid biosynthesis. A total loss of this enzymatic activity as predicted for MRD would lead to a block in the last step of sulfolipid biosynthesis and to the accumulation of precursors, particularly UDP-sulfooquinovose. To test this hypothesis, we incubated cells of wild type and line MRD in the presence of \(^{35}S\) sulfate which was readily taken up by sulfur-starved cells and incorporated into all compounds containing sulfur. We optimized the extraction procedure toward the isolation of sugar nucleotides (20) and separated the water-soluble compounds contained in the extracts by thin layer chromatography using conditions known to be optimal for UDP-sulfooquinovose (7). Using this approach we could detect a \(^{35}S\)-labeled compound of much greater abundance in extracts of MRD cells (Fig. 5). Densitometric scanning of TLC lanes as shown in Fig. 5 indicated that the relative amount of the labeled compound was increased 8–10-fold in the mutant. With the goal of performing structural analysis we attempted to purify a larger amount of this compound. However, even after considerable scale up we were not able to detect the compound by means other than autoradiography. In addition, after optimization of the labeling experiment still only 0.001% of the labeled sulfur in the culture medium (1 mCi/500 ml) was found in this compound. Based on the specific radioactivity of sulfate used in the experiment (100 mCi/mmol), we could estimate that approxi-

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mately 10 nmol of the compound were produced in a 500-ml culture of the MRD mutant containing approximately 0.5 g of wet cells. Purification of sufficient unlabeled material for detailed structural elucidation of this compound employing different methods of spectroscopy would require a considerable increase in the scale of the experiment. As a more feasible alternative, we isolated the radiolabeled compound in sufficient amount to permit an identification based on chromatographic behavior and substrate properties for defined enzymes.

The Accumulating Compound Co-chromatographs with Synthetic UDP-sulfoquinovose—Using three different thin layer chromatography systems as described under “Materials and Methods,” we were able to demonstrate that the Rₚ values for the labeled compound accumulating in MRD and synthetic UDP-sulfoquinovose were identical (data not shown). Furthermore, we employed high performance liquid chromatography to resolve sulfosugar nucleotides according to their constituent base. Four synthetic nucleoside sulfoquinovoses were mixed with a small sample of the labeled compound and analyzed. Given that the radio detector signal is slightly lagging behind due to the experimental setup, a clear match of retention times between the radio-detector signal indicative for the unknown compound and the UV detector signal for synthetic UDP-sulfoquinovose was apparent (Fig. 6).

The nucleotide structure of the labeled compound was confirmed by cleavage with specific enzymes and subsequent identification of the cleavage products by co-chromatography with standards. The labeled compound was mixed with an excess of synthetic unlabeled UDP-sulfoquinovose and treated either with nucleotide pyrophosphatase or a combination of nucleotide pyrophosphatase and alkaline phosphatase. The nucleotide pyrophosphatase degrades sugar nucleotides releasing a sugar phosphate and a nucleoside monophosphate. In the presence of alkaline phosphatase, these products are converted to free sugar and the corresponding nucleoside. Following the enzymatic hydrolysis, the products were analyzed by high performance liquid chromatography. The elution of the base containing part of synthetic UDP-sulfoquinovose was monitored on-line by UV absorption, and the radioactive portion of the unknown compound by radio detection. Treatment with nucleotide pyrophosphatase resulted in complete disappearance of the UV signal for UDP-sulfoquinovose and the overlapping radio signal characteristic for the unknown compound. Concomitantly, a UV signal for uridine monophosphate and a radio signal for a radioactive compound eluting after uridine monophosphate appeared (data not shown). Combined treatment with nucleotide pyrophosphatase and alkaline phosphatase resulted in a UV signal for uridine and a radio signal for a new compound escaping UV detection and eluting ahead of uridine (data not shown). Under the

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![Figure 3](image-url)  **Fig. 3.** Separation of ³⁵S-labeled lipids from wild type and MRD mutant line by thin layer chromatography. Approximately equal amounts of total lipids were loaded in case of undiluted extracts (undil.). In addition, 10-, 100-, and 1000-fold dilutions of the wild type extracts were loaded for estimation of the reduction of sulfolipid in the mutant extracts. Radiolabeled lipids were visualized by autoradiography. F., solvent front; O, origin; SL, sulfolipid; U, unidentified compound.

![Figure 4](image-url)  **Fig. 4.** Complementation analysis of sqd mutants using different subfragments of the sulfolipid operon. The chemically induced mutants carrying defects in the sqdB gene (CHB16) or the sqdC gene (CHB18) as well as the plasmids were constructed during a previous study (10). The open arrows indicate the sqd genes. Vector sequences are drawn with a thick line; the solid arrows indicate the position and orientation of the cytochrome c₂ promotor (Pyc) driving the expression of some subfragments of the operon. The gray box marks the position of the open reading frame of sqdD. The result of the complementation analysis is indicated as presence (+) or absence (−) of sulfolipid in lipid extracts separated by TLC. The gray box highlights the result for the MRD mutant line. *, point mutation; †, insertion mutation. Restriction sites are as described in Figs. 1 and 2.
conditions employed, a complete conversion of substrates was observed for the two different reactions.

To demonstrate that the radioactive hydrolysis products co-chromatographed with the expected compounds, synthetic sulfoquinovosyl 1-phosphate and sulfoquinovose (40 μg each) were added after termination of the reactions to the hydrolysis mixtures and subjected to high performance liquid chromatography. For this purpose the effluent was manually collected, and the fractions were monitored for the presence of sulfoquinovose (colorimetry with anthrone) and radioactive compounds. Nucleotide pyrophosphatase released a labeled product co-chromatographing with sulfoquinovosyl 1-phosphate (Fig. 7, right traces). The additional treatment with alkaline phosphatase produced a labeled product co-chromatographing with sulfoquinovose (Fig. 7, left traces). Taken together, these results present strong biochemical evidence for the identity of the unknown compound with UDP-sulfoquinovose.

Chloroplast Membranes from Spinach Incorporate the 35S-Labeled Part of the Compound into Sulfolipid—To demonstrate that the compound accumulating in MRD is a precursor of sulfolipid biosynthesis, we initially incubated French press extracts from R. sphaeroides wild type cells in the presence of a small aliquot of the compound. However, using this crude system we could not detect any incorporation of label into sulfolipid. We therefore used isolated spinach membranes which contain a well characterized UDP-sulfoquinovosyl diacylglycerol sulfoquinovosyltransferase activity (8). Incubation of these membranes with a small aliquot of the labeled compound accumulating in MRD resulted in the formation of labeled sulfolipid (Fig. 8). As a control, we incubated the membranes in parallel with UDP-[U-14C]galactose to monitor the formation of monogalactosyldiacylglycerol. Given the well characterized substrate specificity of the spinach sulfoquinovosyltransferase, this result provides further biochemical evidence that the compound accumulating in MRD is the sulfolipid precursor UDP-sulfoquinovose.

DISCUSSION

Our objective was to test whether the open reading frame flanked by sqdB and sqdc in the sulfolipid operon of R. sphaeroides represents a new gene crucial for sulfolipid biosynthesis. Our strategy was to disrupt the open reading frame without affecting the expression of the neighboring sulfolipid genes. As a precautionary measure we designed an inactivation cassette containing the outward reading cytochrome c2 promoter from R. capsulatus at the 3′-end of a neomycin phosphotransferase.
gene. Insertion of this cassette in proper orientation resulted in specific inactivation of the targeted open reading frame which was confirmed by complementation analysis. However, further experiments will be required to decide whether this cassette can be used in a general way for nonpolar mutagenesis in R. sphaeroides. The specific disruption of the targeted open reading frame led to sulfolipid deficiency thereby defining a new sulfolipid gene, which we tentatively designated sqdD. It is the fourth gene of R. sphaeroides which was shown to be crucial for sulfolipid biosynthesis using mutational analysis combined with genetic complementation (9,10). Three of the genes, sqdB, sqdC, and sqdD are located in one transcriptional unit (Fig. 4).

Of all four genes, only for sqdD a clearer picture emerges about the possible function of the gene product. First, the predicted amino acid sequence encoded by this gene shows sequence similarity to a UDP-glucose-dependent glycosyl transferase (10), an enzyme with functional analogy to the UDP-sulfoquinovose-dependent sulfoquinovosyltransferase known to catalyze the last step of sulfolipid biosynthesis in spinach chloroplasts (8). Second, the MRD mutant described here which is unable to express sqdD accumulates a 35S-labeled compound tentatively identified as UDP-sulfoquinovose. Since we were unable to purify a sufficient amount of unlabeled compound for structural analysis by spectroscopy, we alternatively applied two established experimental approaches to determine the possible chemical nature of the 35S-labeled compound. One line of evidence was based on co-chromatography with synthetic standards, the second on the conversion of the UDP-sulfoquinovose by enzymes with well defined substrate specificities.

The compound clearly co-chromatographed in the three different thin layer systems tested, as well as in the high performance liquid chromatography system with synthetic UDP-sulfoquinovose. It was completely cleaved by nucleotide pyrophosphatase, confirming a nucleotide structure. The resulting 35S-labeled cleavage product co-chromatographed with sulfoquinovose 1-phosphate. Further dephosphorylation using alkaline phosphatase resulted in co-elution of the labeled cleavage product with sulfoquinovose. These independent observations were all in agreement with a UDP-sulfoquinovose structure for the compound accumulating in MRD. In addition, the labeled portion of the molecule was incorporated into a lipophilic compound co-chromatographing with sulfolipid following the incubation with isolated spinach chloroplast membranes. These membranes contain a sulfoquinovosyltransferase highly specific for UDP-sulfoquinovose as compared to other nucleotide sulfoquinovosides (7). Taken together, these results provide convincing evidence for a UDP-sulfoquinovose structure of the compound accumulating in the sqdD insertional mutant MRD. More than 30 years ago, a sulfur-containing sugar nucleotide, presumably UDP-sulfoquinovose, was observed by paper chromatography of Chlorella extracts (6), a result which has not been reproduced since.

To address the question whether sqdD encodes the sulfoquinovosyltransferase of R. sphaeroides we tried to express this gene in E. coli in a functional form using different strategies. Currently, all our attempts have failed. Although the accumulation of presumably UDP-sulfoquinovose in a sqdD-inactivated strain suggests the idea that the sqdD protein at least may be involved in some aspect of the transfer reaction, it remains to be seen whether the holoenzyme requires additional proteins or factors missing in E. coli. The MRD mutant line provides a new tool for sulfolipid research. Since it apparently accumulates UDP-sulfoquinovose it can be utilized as a source for radiolabeled UDP-sulfoquinovose, particularly if the yield can be increased. Currently, the multistep chemical synthesis of UDP-sulfoquinovose (7) represents no feasible alternative to the biochemical preparation of labeled UDP-sulfoquinovose with high specific activity as required for biochemical studies.

The accumulation of UDP-sulfoquinovose in a sulfolipid-deficient mutant of R. sphaeroides and the incorporation of the labeled part of this molecule into sulfolipid by isolated spinach chloroplast membranes provide further experimental evidence for the hypothesis that the head group of sulfolipid is derived from UDP-sulfoquinovose in photosynthetic bacteria and higher plants. Whether the transfer reaction itself proceeds in the same way in photosynthetic bacteria and plants must await further experiments. The question for the pathway of sulfolipid biosynthesis can be reduced to the question for the biosynthesis of UDP-sulfoquinovose. Further analysis of the proteins encoded by the four known sulfolipid genes of R. sphaeroides, particularly in vitro reconstitution experiments, may provide the answer to this question in the future.

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![Fig. 8. Separation of lipid extracts from spinach chloroplast membranes incubated with the 35S-labeled unknown compound or UDP-[U-35S]galactose.](Image) Lipids were visualized by A, autoradiography and by B, charring. F, solvent front; MGD, monogalactosyl diacylglycerol; DGD, digalactosyl diacylglycerol; O, origin; SL, sulfolipid.)