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# Isolation and Genetic Complementation of a Sulfolipid-Deficient Mutant of *Rhodobacter sphaeroides*

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**All photosynthetic organisms are thought to contain the sulfolipid 6-sulfo-α-D-quinovosyl diacylglycerol. However, the pathway of sulfolipid biosynthesis has not been elucidated, and the functional or structural significance of this lipid is not known. Mutants of *Rhodobacter sphaeroides* deficient in sulfolipid accumulation were isolated by directly screening for altered sulfolipid content. The mutants had no apparent phenotype except for the sulfolipid deficiency. A gene, designated *sqdA*, which complemented one of the mutations was isolated and characterized. The putative *sqdA* gene product is a protein with a molecular mass of 33.6 kDa that has no sequence similarity to any enzyme of known function.**

Since the discovery (7) and structural elucidation (6) of sulfoquinovosyl diacylglycerol (sulfolipid) by Benson and colleagues over 30 years ago, relatively little progress has been made towards the elucidation of the biosynthetic pathway and the function of this unusual lipid. This is somewhat surprising, since the occurrence of substantial amounts of the sulfolipid in all photosynthetic organisms examined makes it one of the most abundant sulfur-containing organic compounds. The proportion of sulfolipid in total, ether-extractable lipids varies from 2.6% in *Rhodobacter sphaeroides* up to 18.6% in the brown alga *Fucus vesiculosus* (27) and can account for as much as 40% of the glycolipids in marine red algae (11). Leaves of higher plants usually contain approximately 5% sulfolipid, which is confined to the photosynthetic lamellae. The association of the plant sulfolipid with photosynthetic membranes has led to the assumption that this lipid plays some role in photosynthesis (3). However, experimental evidence supporting this idea is lacking.

Many of the proposed pathways for sulfolipid biosynthesis have been derived from knowledge about photosynthetic carbon assimilation. Several possible pathways for the biosynthesis of sulfoquinovosyl diacylglycerol have been proposed on the basis of biosynthetic parsimony rather than the availability of any direct evidence (16, 23). Either adenosine phospho-sulfate (in higher plants) or phosphoadenosine phospho-sulfate (in photosynthetic bacteria) is generally believed to be the donor of the sulfur residue in sulfoquinovosyl diacylglycerol, but the nature of the carbon compound accepting the sulfur is not known. Indirect evidence obtained by Heinz and coworkers (17) suggested that the last step of sulfolipid biosynthesis involves the transfer of the sulfoquinovose sugar from UDP-sulfoquinovose to diacylglycerol by a membrane-bound transferase. However, the sequence of reactions leading to the formation of UDP-sulfoquinovose remains unknown.

The photosynthetic purple nonsulfur bacterium *R. sphaeroides* was previously reported, on the basis of chromatographic $R_f$ values, to contain sulfoquinovose diacylglycerol (34). A recent comparison of sulfolipids isolated from spinach and *R. sphaeroides* by mass spectroscopy techniques confirmed the identity of the sulfoquinovose head group of the bacterial sulfolipid (14). Therefore, we have applied the common techniques of bacterial genetics to the problem of sulfolipid synthesis in *R. sphaeroides*. It was hoped that specific blocks in the pathway of sulfolipid biosynthesis would lead to the accumulation of biosynthetic precursors which could be isolated and identified.

Here, we describe the isolation and preliminary characterization of a mutant deficient in sulfolipid biosynthesis. By complementation of this mutant, a novel gene involved in sulfolipid biosynthesis was isolated and characterized.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are shown in Table 1. The expression vector pCHB500 (Fig. 1) was constructed by inserting the promoter region upstream of the *cycA* gene from *Rhodobacter capsulatus* into the *Hin*dIII and *Pst*I sites of pRK415. The region from nucleotide 1 through nucleotide 354 of the sequence of the *cycA* gene (10) on plasmid pSH3 was cloned by polymerase chain reaction employing synthetic oligonucleotides containing the desired *Hin*dIII or *Pst*I sites.

**Media and growth conditions.** Cell cultures were grown in the malate-basal salts medium described by Ormerod et al. (24) or in Sistrom's succinate-basal salts medium (31, 32). Agar plates (1.5% agar) were incubated either in the dark at 30°C in air or in the light (100 microeinsteins $m^{-2}$ $s^{-1}$) at 30 to 35°C in an atmosphere of 5% $CO_2$ and 95% $N_2$. When required, 0.8 μg of tetracycline per ml was added to agar plates containing Sistrom's medium. Aerobic chemoheterotrophic, liquid cultures inoculated with a single colony were incubated at 30°C with shaking in Erlenmeyer flasks. Anaerobic photoheterotrophic, liquid cultures were grown in tightly closed, filled 200-ml bottles in the light (100 microeinsteins $m^{-2}$ $s^{-1}$) at 30 to 35°C. The cultures in the bottles were mixed once or twice a day by shaking.

For growth of mutagenized cells of *R. sphaeroides* on complete medium, Z-broth (26) was used. Plates containing solidified medium were incubated in darkness in an incubator at about 35°C; liquid cultures were grown in darkness in a shaker at 35°C.

*Escherichia coli* strains were grown on LB medium. When required, tetracycline (10 μg/ml), kanamycin (50 μg/ml), or ampicillin (100 μg/ml) was added to the medium.

**Mutagenesis of *R. sphaeroides*.** To 20 ml of a *R. sphaeroides* wild-type mid-log-phase culture (Z-broth), crystals of

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Description or construction | Reference |
|---|---|---|
| *R. sphaeroides* 2.4.1 | Wild type | R. L. Uffen |
| *R. sphaeroides* CHB17 | Sulfolipid deficient, *sqdA* mutant | This study |
| *E. coli* HB101 | *proA2 recA13 mcrB* | 22 |
| *E. coli* DH5αF′ | F′*endA1 recA1* Δ(*lacZYA-argF*)*U169* [φ80d*lac*Δ(*lacZ*)M15] | 28 |
| *E. coli* MM294 | *endA1 hsdR17 thi-1* | 2 |
| pBS-KS$^+$, pBS-KS$^-$ | pBluescript vector Amp$^r$ | Stratagene |
| pRK2013 | Kan$^r$ Tra$^+$ RK2-ColE1$_{rep}$ | 13 |
| pLA2917 | Kan$^r$ Tc$^r$ *cos* | 1 |
| pRK415 | Tc$^r$ RK2$_{rep}$ | 19 |
| pSH3 | Amp$^r$ plasmid carrying the *cycA* gene of *R. capsulata* | F. Daldal |
| pCHB500 | Tc$^r$ expression vector for *R. sphaeroides* derived from pRK415 and pSH3 | This study |
| pCHB1701 | pLA2917-derived cosmid complementing the *R. sphaeroides sqdA* mutant | This study |
| pCHB17011, pCHB17012 | Tc$^r$ *sqdA*$^+$; *Sau*3A-partial-digest subclones of pCHB1701 in pLA2917 | This study |
| pCHB17B20-D$^+$, -A$^+$ | Amp$^r$; 2.1-kb *Bam*HI fragment of pCHB17012 in pBS-KS$^+$ (both orientations) | This study |
| pCHB1741, pCHB1742 | Tc$^r$ *sqdA*$^+$; 2.1-kb *Xba*I, *Eco*RI fragments of pCHB17B20-D$^+$ and pCHB17B20-A$^+$ cloned into pCHB500 | This study |
| pCHB1751, pCHB1752 | Tc$^r$ *sqdA*$^+$; 1.2-kb *Bam*HI fragment of pCHB17B20-A$^+$ cloned in both orientations into pCHB500 | This study |
| pCHB17NS-I, -II | Amp$^r$; partial 1.74-kb *Stu*I, *Nae*I fragment of pCHB17B20 cloned in both orientations into pBS-KS$^+$ | This study |
| pCHB1761 | Tc$^r$ *sqdA*$^+$; 1.8-kb *Kpn*I, *Eco*RI fragment of pCHB17NS-I cloned into pCHB500 | This study |
| pCHB1762 | Tc$^r$; 1.8-kb *Kpn*I, *Eco*RI fragment of pCHB17NS-II cloned into pCHB500 | This study |

*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (nitrosoguanidine) were added to obtain a final concentration of 0.1 mg/ml and the culture was incubated at room temperature with vigorous shaking. At various times (0, 10, 20, 30, and 45 min following the addition of the mutagen), 3-ml aliquots were taken and the cells were washed once with 3 ml of Z-broth. Aliquots of the washed cells were plated directly onto agar-solidified Z-broth to establish a killing curve. The washed cultures were incubated for 17 h with shaking at 35°C to allow the cells to replicate two to three times. Aliquots of these cultures were plated onto Z-broth plates containing rifampin (100 μg/ml) or streptomycin (100 μg/ml) to test for the degree of mutagenesis by scoring the frequency of antibiotic-resistant mutants. Additional aliquots were plated onto plain Z-broth plates to determine the total amount of cells in the culture and to initiate the screening for mutants. The remainder of the cultures were brought to 20% (vol/vol) dimethyl sulfoxide and stored at −70°C.

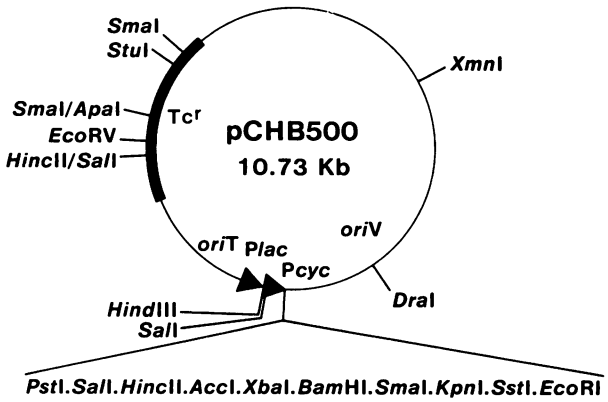


FIG. 1. Circular restriction map of pCHB500. The orientation and location of two promoters, P*lac* (promoter of the *E. coli lac* operon) and P*cyc* (putative *R. capsulatus* promoter of the *cycA* gene), are indicated by arrowheads. The tetracycline resistance gene (Tc$^r$) is indicated by a thick line. The locations of the origin of transfer (*oriT*) and origin of replication (*oriV*) are shown. Restriction sites are indicated. The sites of the polylinker, downstream from the P*cyc* promoter, are shown in an expanded view.

**Screening for polar lipid mutants.** Randomly chosen colonies from a mutagenized population of *R. sphaeroides* cells were streaked as small patches (0.5 by 0.5 cm) on fresh Z-broth plates. Lipids were isolated from these patches by collecting cells onto the wide end of a flat toothpick and swirling the material in 75 μl of chloroform-methanol (1:1, vol/vol) contained in polypropylene microcentrifuge tubes. Following the addition of 25 μl of 1 N KCl–0.2 M $H_3PO_4$, the tubes were vortexed and centrifuged to separate the organic and aqueous phases. A 10-μl aliquot was withdrawn from the lipid-containing lower phase and was directly spotted onto an activated ammonium sulfate-impregnated silica gel thin-layer chromatography (TLC) plate (modified by the method of Kahn and Williams [18]). For this purpose, Baker Si250 silica plates with a preadsorbent layer were prepared by soaking in 0.15 M ammonium sulfate for 30 s followed by air drying to complete dryness. Immediately prior to use, the plates were activated for 2.5 h at 120°C. Activation of ammonium sulfate-treated plates at 120°C produces sulfuric acid, which protonates phosphatidylglycerol, making it less polar. An acetone-benzene-water mixture (91:30:8, vol/vol/vol) was employed as the solvent system. Under these conditions, phosphatidylglycerol was readily separated from the sulfolipid, permitting easy scoring of mutants with an altered lipid composition. Lipids were visualized by spraying the plates with 50% sulfuric acid followed by heating at 160°C for 10 to 15 min to char the lipids.

**Quantitative lipid analysis of mutant and wild-type bacteria by gas chromatography of fatty acid methyl esters.** For each strain, three 50-ml cultures were grown in Sistrom's medium aerobically with shaking at 32°C in the dark. The cells were

centrifuged, suspended in 0.5 ml of water, and extracted by vortexing with 4 ml of chloroform-methanol (1:1, vol/vol). Addition of 1.3 ml of 1 M KCl–0.2 M $H_3PO_4$, vortexing, and centrifugation resulted in phase partitioning of the lipids into the lower chloroform phase. The chloroform phase was removed and concentrated to 0.2 ml by evaporation under a stream of $N_2$. The sample was split, and the material was spotted onto activated (30 min at 110°C) silica TLC plates (Si250; Baker). The plates were developed in two dimensions, first with chloroform-methanol-water (65:25:4, vol/vol/vol) and then with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by volume). Lipids were visualized with iodine vapor, and after desorption of iodine, the spots were individually scraped into 8-ml screw-cap tubes. To the samples, 5 μg of myristic acid methyl ester in 0.1 ml of hexane was added as an internal standard, since only negligible amounts of endogenous myristic acid were found in the bacterial lipids. Fatty acid methyl esters were prepared by addition of 1 ml of anhydrous 1 N methanolic HCl (Supelco) followed by incubation at 80°C for 1 h. Following the addition of 1 ml of 0.95% (wt/vol) KCl, the fatty acid methyl esters were extracted into 1 ml of hexane and then dried to a volume of 0.1 ml. Samples (2 μl each) were injected onto a gas chromatograph (Varian 2000), which was equipped with a 2.4-m column (2-mm inner diameter) packed with 3% SP-2310 and 2% SP-2300 on 100/120 Chromosorb W AW (Supelco). The carrier gas ($N_2$) flow rate was adjusted to 20 ml/min, and the column temperature was set for 2 min at 180°C, increasing to 200°C over 10 min, and 4 min at 200°C. The fatty acid methyl esters were detected by a flame ionization detector, and the data were integrated by a Spectra Physics integrator. To calculate the relative amounts of the eight polar lipids included in the analysis, the amount of fatty acids contained in each lipid was calculated. The validity of calculation was based on the assumption that each of the lipids, including the unidentified lipids, contained two fatty acids per molecule and that the different lipids had a similar fatty acid composition. A total of six samples (two for each of the three cultures) per strain were analyzed, and means and standard deviations were calculated.

**Time course for the incorporation of $^{35}$S-sulfate into proteins and sulfolipid.** Cells (grown photoheterotrophically in Ormerod's medium) from a 2-ml late-log-phase culture were collected by centrifugation and resuspended in eight 2-ml microcentrifuge tubes holding low-sulfate Ormerod's medium containing 10 μCi of $^{35}$S-sulfate each (specific activity adjusted to 0.5 μCi/nmol). These cultures were grown in the dark at 30°C. One of each of the eight parallel cultures was analyzed at appropriate times after addition of the label. Following centrifugation, the cells were extracted with 0.5 ml of methanol-chloroform-formic acid-water (12:5:1:2, vol/vol/vol). The extracts were stored at −20°C. Proteins and cell debris were pelleted by centrifugation. The supernatant was transferred to a new tube, and the pellet was reextracted with 0.25 ml of methanol-formic acid-water (40:1:59, vol/vol/vol). Following centrifugation, the supernatants from the two extractions were combined and phase partitioned by the addition of 0.15 ml of chloroform and 0.05 ml of water. The chloroform phase contained the labelled sulfolipid, which was further analyzed by TLC on ammonium sulfate plates. The sulfolipid band was scraped from the plate, and the radioactivity was determined by scintillation counting. The pellets, containing most of the protein and cell debris, were suspended in 0.05 ml of 0.1 N NaOH and incubated at 37°C for 30 min. The samples were diluted with 0.45 ml of $H_2O$, and 0.1-ml portions were used for protein determination by using the Pierce bicinchoninic acid protein assay according to the manufacturer's instructions. The radioactivity in 0.01-ml samples was measured by scintillation counting.

**Construction of the cosmid library.** High-molecular-weight DNA from wild-type *R. sphaeroides* cells grown photoheterotrophically in Sistrom's medium was prepared by a modified procedure originally designed for yeast chromosomes (9), except that zymolase was replaced by lysozyme (2 mg/ml) to prepare spheroplasts.

Aliquots (ca. 5 to 10 μg each) from this DNA preparation were partially digested with *Sau*3AI, and fragments in the size range from 25 to 30 kb were ligated into the *Bgl*II site of the cosmid vector pLA2917. The resulting cosmids were packaged in vitro and transfected into *E. coli* HB101. Cell lines containing the cosmids were selected on Luria broth plates containing tetracycline (10 μg/ml). Individual colonies were transferred to 96-well plates, brought to 20% (vol/vol) dimethyl sulfoxide, and stored frozen at −70°C.

**Triparental mating and complementation assay.** An aliquot (5 ml) from a mid-log-phase culture grown photoheterotrophically (Sistrom's medium) was diluted with 20 ml of Sistrom's medium, and 100 μl of a 50-fold-concentrated mid-log-phase culture of washed *E. coli* MM294 cells containing the helper plasmid pRK2013 was added. This mixture was poured over a sterile cellulose acetate filter (MSI; 9-cm diameter) under suction. Following the complete removal of the liquid, the underside of the filter was blotted onto sterile Whatman no. 2 filter paper and placed onto a Luria broth agar plate. By using a sterilized pronging device matching 48 wells of a 96-well microtiter plate, frozen material from the −70°C cosmid library stocks was transferred directly onto the filter containing the lawn of *sqdA* mutant and helper cells. The plates, containing the filters carrying the three parent cell lines, were incubated for 6 h at 30°C in the dark prior to transfer of the filters onto agar plates containing Sistrom's medium with tetracycline (0.8 μg/ml). Exconjugants became visible after 3 to 4 days of incubation for photoheterotrophic growth as described above. The sulfolipid phenotype of the exconjugants was tested by extraction of the lipids, followed by TLC analysis on ammonium sulfate-impregnated plates.

**Restriction analysis and agarose gel electrophoresis.** For detailed restriction maps, 1 μg of CsCl gradient-purified DNA was digested in 20 μl of the recommended buffer for 1 to 2 h at the recommended temperature. Gel electrophoresis was generally performed by using 0.8% (wt/vol) agarose gels in combination with Tris-borate–EDTA buffer as described previously (22).

**Southern analysis.** Probes were labelled by random priming (12). DNA fragments separated on agarose gels were blotted onto Hybond-$N^+$ (Amersham) nylon membranes by the alkaline transfer method, and the DNA was hybridized by the method of Reed and Mann (29).

**Sequencing.** DNA sequences were determined by employing the chain termination reaction method (30). In reaction mixtures, single-stranded templates (about 200 ng per reaction) were sequenced by using [α-$^{35}$S]dATP as the labelled substrate (8) and modified T7 DNA polymerase (Sequenase [33]). To reduce sequencing artifacts caused by the high GC content of *R. sphaeroides* DNA, GTP was replaced by 7-deaza-dGTP (4) in the reactions. The labelled DNA fragments were separated on 6% polyacrylamide–8 M urea slab gels (25).

**Preparation of synthetic oligonucleotides.** Oligonucleotides used as sequencing primers were synthesized on an Applied Biosystems 380A DNA synthesizer by the phosphoramidite

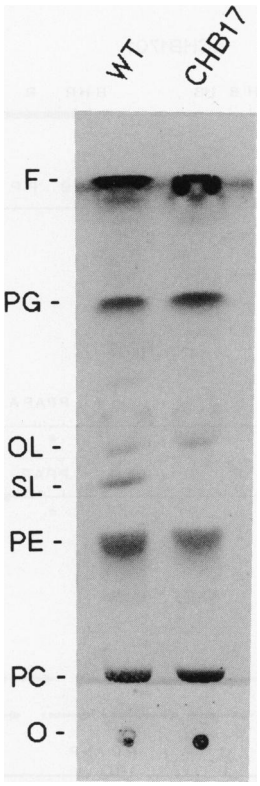


FIG. 2. Separation of lipids from the wild type (WT) and from the *sqdA* mutant (CHB17) on ammonium sulfate plates. The bands were visualized by charring. F, solvent front; PG, phosphatidylglycerol; OL, ornithine lipid; SL, sulfolipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; O, origin.

method (5). Following "trityl-on" synthesis, the oligonucleotides were purified on $C_{18}$ reverse-phase cartridges (SEP-PAK; Waters).

**Nucleotide sequence accession number.** The DNA sequence was deposited in GenBank with the accession number M83823.

## RESULTS

**Isolation of a mutant deficient in the biosynthesis of the plant sulfolipid in *R. sphaeroides*.** Mutagenesis of *R. sphaeroides* with nitrosoguanidine for 45 min increased the frequency of mutations to resistance to the antibiotics rifampin and streptomycin and the occurrence of pigment mutations (e.g., dark red colonies as opposed to wild-type pink colonies) by approximately 100-fold over the spontaneous frequencies in wild-type cultures. Under these conditions, the survival rate was less than 1%. The lipid composition in extracts of 1,512 randomly chosen cell lines from this mutagenized population was visually examined by TLC using ammonium sulfate-impregnated plates, which allowed for the separation in one dimension of all the major polar lipids found in *R. sphaeroides* (Fig. 2). These major polar lipids include phosphatidylcholine, phosphatidylethanolamine, sulfolipid, ornithine lipid, and phosphatidylglycerol (15, 34).

Of the 1,512 cell lines tested, 11 putative mutants showing reduced levels of sulfolipid were identified in the primary screening. Three of these mutants were unable to grow photoheterotrophically on Ormerod's minimal medium and have not been further analyzed. The remaining putative mutants were examined for their ability to incorporate $^{35}$S-sulfate into sulfolipid in comparison to the wild type. Four mutant lines incorporated reduced but significant levels of sulfate into sulfolipid as compared with the wild type, and the remaining four mutant lines showed almost no incorporation of labelled sulfate into sulfolipid. In order to explore the basis for the defect in sulfolipid biosynthesis, we chose to focus on the detailed analysis of one line, CHB17, which had very low levels of sulfolipid but was able to grow photoheterotrophically. A one-dimensional separation of the lipids of mutant line CHB17 and the wild type is shown in Fig. 2. The mutated gene responsible for the deficiency in sulfolipid in line CHB17 was provisionally designated *sqdA*.

TABLE 2. Polar lipid composition of the *R. sphaeroides* wild type and *sqdA* mutant[a]

| Lipid | Composition (% by weight ± standard error) ($n$ = 6) in: | |
|---|---|---|
| | Wild type | *sqdA* mutant |
| Sulfolipid | 5.4 ± 0.4 | 0.7 ± 0.2 |
| Phosphatidylcholine | 15.4 ± 0.8 | 20.0 ± 1.5 |
| Phosphatidylglycerol | 18.7 ± 0.9 | 24.2 ± 1.4 |
| Phosphatidylethanolamine | 44.8 ± 0.9 | 38.4 ± 2.1 |
| Ornithine lipid | 1.8 ± 0.5 | 3.2 ± 1.4 |
| Unidentified lipid I | 1.7 ± 0.3 | 2.0 ± 0.4 |
| Unidentified lipid II | 12.0 ± 1.1 | 11.6 ± 1.3 |

[a] Cells were grown chemoheterotrophically.

**Primary phenotype of the *sqdA* mutant.** For a quantitative comparison of the lipid compositions of the wild type and the *sqdA* mutant, lipid extracts were separated in two dimensions on silica TLC plates. Lipids were identified by isolation of the spots from the plates, followed by cochromatography with standards on TLC plates. No sulfolipid spot could be detected by charring of the separated lipids from the *sqdA* mutant samples. To quantify the lipids, the iodine-stained spots, representing more than 95% of the polar lipids, were isolated from the plates and fatty acid methyl esters were prepared and quantified by gas chromatography (Table 2). The result of the experiment indicated that the sulfolipid content in the *sqdA* mutant was reduced to about 10% of the wild-type level. The amounts of phosphatidylcholine and phosphatidylglycerol were slightly increased in the mutant. The residual amounts of sulfolipid consistently detected indicated that the *sqdA* mutant is slightly leaky.

To determine whether the *sqdA* mutant harbors a defect in the general sulfur assimilation pathway, which in turn could affect protein biosynthesis, rates of incorporation of $^{35}$S-sulfate into proteins and sulfolipid were compared (Fig. 3). The mutant and the wild type incorporated labelled sulfate into protein at similar rates, whereas the labelling of sulfolipid was greatly reduced in the mutant. The increase in labelled protein was paralleled by the increase in total protein as determined by a colorimetric protein assay (data not shown). Since the biosynthesis of cysteine and methionine was not affected, the *sqdA* mutant appears to be specific for sulfolipid biosynthesis. The result was in agreement with the fact that the *sqdA* mutant can grow photoheterotrophically on Ormerod's and Sistrom's media, which do not contain any reduced sulfur compounds. However, its growth rate is reduced compared with that of the wild type (data not shown). Whether this reduction in growth rate is related to

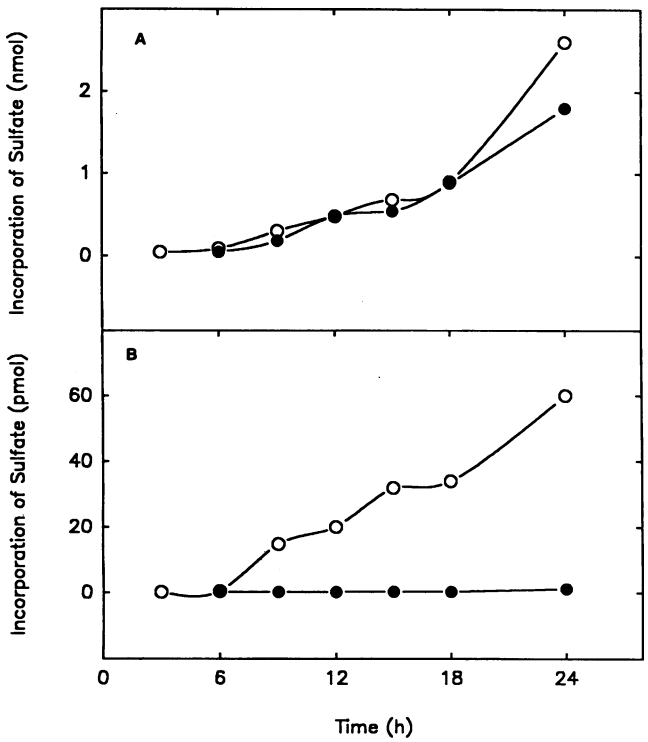


FIG. 3. Time course for the incorporation of $^{35}$S-labelled sulfate into protein (A) and sulfolipid (B) by the wild type (○) and by the *sqdA* mutant (●).

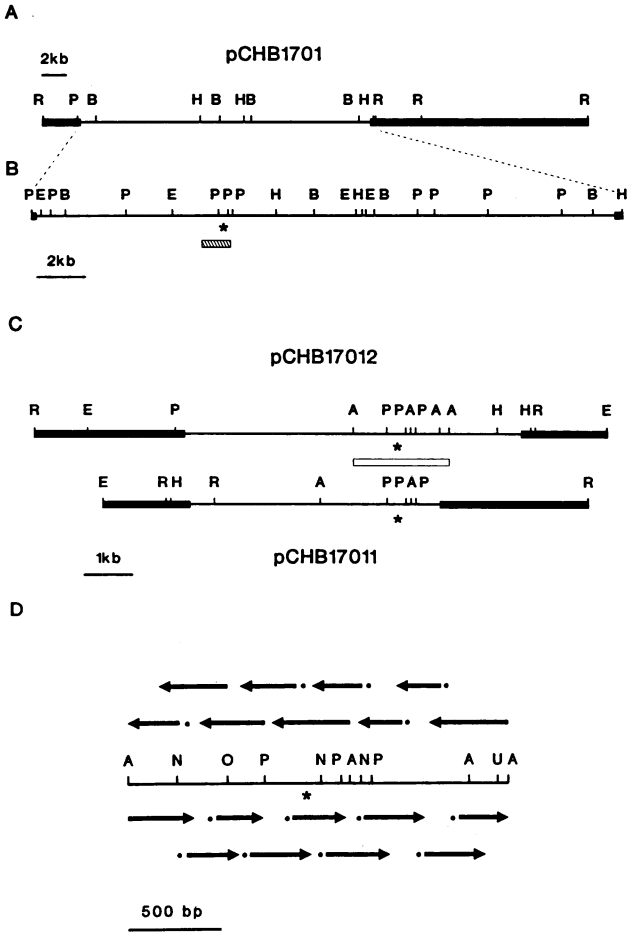


FIG. 4. Restriction map for the cosmid clone pCHB1701 and its derivatives. (A) Overview showing insert and vector. (B) Expanded view of the insert of pCHB1701. The location of the smallest subclones complementing the *sqdA* mutant (pCHB1751, pCHB1752) is indicated (▨). (C) Restriction map of pCHB17011 and pCHB17012, which were aligned to match the common region around the 0.4-kb *Pst*I fragment (*). The location of the pCHB17012 *Bam*HI fragment giving rise to the pCHB17B20 subclone series is indicated (▭). (D) Strategy for sequencing pCHB17B20. Only the 2.1-kb *Bam*HI fragment from pCHB17012 is shown. Sequencing runs (arrows) primed with synthetic oligonucleotides are preceded by a dot. Restriction sites: A, *Bam*HI; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Nae*I; O, *Xho*I; P, *Pst*I; R, *Eco*RV; U, *Stu*I. The pLA2917 vector part is drawn with a thicker line. The location of the 0.4-*Pst*I fragment internal to the *sqdA* gene is indicated (*).

the sulfolipid phenotype is unclear at this point of the analysis because it could be caused by background mutations unrelated to the sulfolipid deficiency.

**Isolation of the *sqdA* gene by complementation.** A 1,700-member cosmid library from wild-type *R. sphaeroides* DNA was constructed by inserting 25- to 30-kb fragments into the *Bgl*II site internal to the neomycin phosphotransferase gene of the 21-kb cosmid vector pLA2917 (1). Given the genome size of approximately $5 \times 10^6$ bp, this library should cover the genome four to five times. Individual cosmid clones were transferred into the *sqdA* mutant by triparental mating. Testing of about 450 exconjugants for restoration of the sulfolipid deficiency by TLC of the lipids yielded two complementing cosmid clones, one of which (pCHB1701) was further analyzed. A complete restriction map of the complementing cosmid clone pCHB1701 (Fig. 4A and B) was prepared by subcloning smaller insert fragments in combination with conventional restriction and Southern analysis.

To localize the *sqdA* gene on the approximately 23- to 25-kb insert of pCHB1701, the complete clone was partially digested with *Sau*3A and 2- to 3-, 3- to 4-, and 4- to 6-kb fragments were cloned into the *Bgl*II site of pLA2917. The subclones were individually mated into the *sqdA* mutant, and two complementing clones (pCHB17011 and pCHB17012) were isolated. Since the size of the insert of both clones was about twice the length of the fragments used in their construction, it appeared that these clones each contained two independent inserts. Comparison of the two clones by restriction and Southern analysis revealed a common region centering around a 0.4-kb *Pst*I fragment (Fig. 4C). This fragment was used to probe DNA cut with *Bam*HI from the two subclones, the original cosmid clone and genomic DNA from *R. sphaeroides* (Fig. 5). A single 1.9-kb fragment could be detected for pCHB17011, pCHB1701, and genomic DNA. However, in pCHB17012 this *Bam*HI fragment was truncated to 1.2 kb because of the subcloning procedure. A 2.1-kb *Bam*HI fragment obtained by partial digestion of pCHB17012, which covered the region common to the two complementing subclones of pCHB1701 (Fig. 4C), was subcloned into pBluescript to give rise to pCHB17B20 and subjected to sequence analysis.

**Sequence analysis and determination of the open reading frame for the *sqdA* gene.** The insert of pCHB17B20, which was completely sequenced on both strands (Fig. 4D), had a GC content of 70.0% (Fig. 6), which is in agreement with values typically observed for *R. sphaeroides* DNA. Analysis

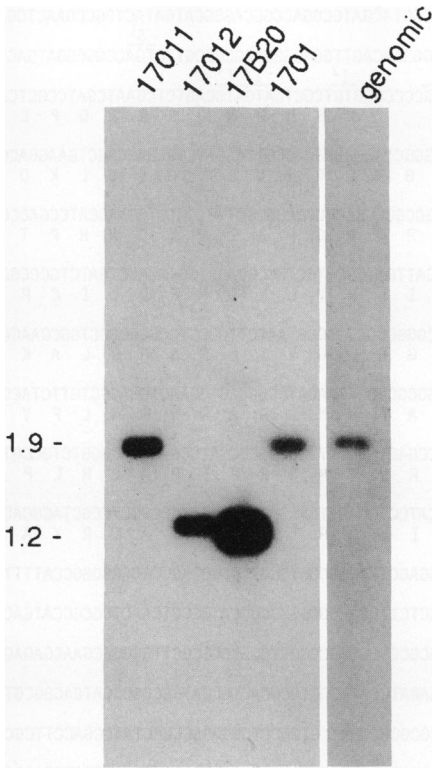


FIG. 5. Genomic DNA and various subclones of pCHB1701 probed with a portion of the *sqdA* gene. The DNA (1 μg of plasmids or 5 μg of genomic DNA) was cut with *Bam*HI and probed with a 0.4-kb *Pst*I fragment internal to the *sqdA* gene. The sizes (in kilobases) of the two hybridizing fragments are indicated.

of the sequence for open reading frames (ORFs) revealed three large ORFs (Fig. 7), which could encode proteins of about 33 kDa (ORF1), 26 kDa (ORF2) and 32 kDa (ORF3). However, complementation analysis of deletion clones, as described below, excluded two of the three ORFs (ORF2 and ORF3). The maximum size of the third ORF (ORF1) was found to be determined by the position of two in-frame stop codons ($S^1$ and $S^2$, Fig. 6). Downstream of the first stop codon, four possible in-frame initiation codons were located, of which only the second, a GTG codon ($I^2$, Fig. 6), was preceded by a putative ribosome-binding site. This putative ribosome-binding site showed perfect sequence complementary to the 3′ end of the *R. sphaeroides* 16S rRNA, as well as correct spacing, as compared with the ribosome-binding sites from other *R. sphaeroides* genes. The presence of a sequence satisfying the requirements for a correctly spaced ribosome-binding site only upstream of the second initiation codon suggested that a GTG codon 7 bp in the 3′ direction was the best candidate for the translational initiation site of the *sqdA* gene. Although the usual initiation codon is ATG, the use of GTG has been observed previously (20).

To directly determine which of the ORFs in the 2.1-kb *Bam*HI fragment of pCHB17012 represented the *sqdA* gene, the complete insert, as well as deletion fragments of pCHB17B20, was cloned in both orientations behind a promoter to create transcriptional fusions, which would result in transcription of the various fragments in *R. sphaeroides*. The clone pCHB17B20 by itself could not be employed for complementation analysis, since pBluescript derivatives cannot replicate in *R. sphaeroides*. Instead, the vector used was pCHB500, which was constructed by inserting the promoter region of the cytochrome $c_2$ gene (*cycA*) from *R. capsulata* into the polylinker of pRK415. This clone was tested for its ability to express ORFs in *R. sphaeroides* by insertion of the ORF of chloramphenicol acetyltransferase or neomycin phosphotransferase in both orientations into the polylinker of pCHB500. Only when the ORFs were inserted in the proper orientation was increased resistance against the respective antibiotic observed. Insertion of the complete fragment of pCHB17B20, corresponding to the 2.1-kb *Bam*HI fragment of pCHB17012, into the *Bam*HI site of pCHB500 in both orientations gave rise to clones pCHB1741 and pCHB1742 (Fig. 7), both of which restored the wild-type phenotype when present in the sulfolipid-deficient *sqdA* mutant. This result confirmed that the 2.1-kb fragment of pCHB17012 was indeed capable of complementing the *sqdA* mutant. In addition, these data suggested that the fragment contained a promoter, making the complementation independent of the insert orientation and therefore independent of the presence of the external *cycA* promoter provided by pCHB500.

A 1.2-kb *Bam*HI subfragment of pCHB17B20 extending from the left end of the insert to the *Bam*HI site directly downstream of the stop codon of the putative *sqdA* ORF (Fig. 6) was cloned in both orientations into the *Bam*HI site of pCHB500, giving rise to subclones pCHB1751 and pCHB1752 (Fig. 7). Both clones complemented the *sqdA* mutation, suggesting that the 1.2-kb *Bam*HI subfragment of pCHB17B20 contained a promoter preceding the *sqdA* coding sequence. These two subclones were the smallest clones capable of complementing the *sqdA* mutant. The location of this fragment on the cosmid clone pCHB1701 is shown in Fig. 4B. The 3′ end of one of the three potential ORFs (ORF3, Fig. 7) was not included in this fragment, making it unlikely that it represents the *sqdA* gene. However, there were still two intact ORFs (ORF1 and ORF2, Fig. 7) located on opposite strands of this fragment.

The coding strand for the *sqdA* gene was identified by obtaining orientation-dependent complementation of the *sqdA* mutation by ORF1 but not ORF2 when these sequences were cloned in the proper orientation behind the *cycA* promoter on pCHB500. A *Nae*I site upstream of the putative initiation codon of ORF1 was used to remove most of the upstream sequence of the putative *sqdA* gene (Fig. 7). By using this site, a 1.8-kb partial *Nae*I-*Stu*I fragment from pCHB17B20 was first cloned in both orientations into the *Eco*RV site of pBluescript. The fragment was excised from pBluescript by using *Kpn*I and *Eco*RI and cloned in both orientations into pCHB500, giving rise to pCHB1761 and pCHB1762 (Fig. 7). Because of the cloning procedure, the *Nae*I-*Stu*I fragment was flanked by small stretches of pBluescript polylinker sequence in pCHB500. In a complementation assay, only pCHB1761, as opposed to pCHB1762, was able to restore the wild-type phenotype. In pCHB1761 the *cycA* promoter is located upstream of ORF1, reading in the direction of the putative *sqdA* gene (Fig. 7), while in pCHB1762 the situation is reversed. Therefore, only ORF1, not ORF2, could represent the *sqdA* gene.

The amino acid sequence predicted by translation of the putative ORF representing *sqdA* suggested that the protein has a molecular mass of about 33.6 kDa. A hydropathy analysis conducted by the method of Kyte and Doolittle (21) revealed that the *sqdA* gene product is most likely a soluble protein. To investigate the possible function of the predicted *sqdA* protein, the deduced amino acid sequence was used to screen two protein data banks (NBRF-PIR release 26 and

```
1     GGATCCGGCTCGACCCGCTGAACCCGCAGACGGCGGCGCGCATGTCGACCGCCTTCGAGACCCGGGCCCGCTACGATGCGGACCGGCAGGGCATGATACTGGCCGAACTCGACCGCATCC
                                                                                                          S1
121   TCGCCACGCCGGGCCTCAGCCGCGATCTGGGCGAGATGGCGGGGCGGATGAGGCACGCATGAGCGGGTGGGAACAGTTGGACGGGAAGCGGCGTTCTGACCGGCGGATGACTGCCGAGCG
                                NaeI                  I1                  SD          I2          I3 I4
241   CAGATCGGTGCAAAGGACGGCGCTTGCCGGCCGGGACAACCGGGGCTCATGTCATGCGAGAGCAGGAGGCCCCGGGTGTCGCTCATGATGCAGTCTCGAATCGATCCGCTCATCGAAGAG
1                                                                                                   M  S  L  M  M  Q  S  R  I  D  P  L  I  E  E

361   CGTGCCCCCTGGCTCTTCTCCGGCCGTCCCCATCATGAAGCCTCGCGCAGACTTCTGTGCCGCGTTCTGGGCTACGACCGCACCGTCGAGATCGGAGAGCAGCTGAAGGACGATCCGACG
16     R  A  P  W  L  F  S  G  R  P  H  H  E  A  S  R  R  L  L  C  R  V  L  G  Y  D  R  T  V  E  I  G  E  Q  L  K  D  D  P  T

481   CCCGAGATCATGCGCCGCGTGGCGCAGATGATCGCGCGGGACGTAGAGGTCTCGGGTCTCGAGAACCTGCCGCGCAACGGCCCGGCGCTGGTCGTCTGCAACCATCCGACCGGCATCGCC
56     P  E  I  M  R  R  V  A  Q  M  I  A  R  D  V  E  V  S  G  L  E  N  L  P  R  N  G  P  A  L  V  V  C  N  H  P  T  G  I  A

601   GACGGGGTGATCCTCCACCACATCCTCGCGCCCCTGCGGCCCGATCTCTTCTTCTACGCCAACCGCGACATTCTTCGCGTGCTGCCGCAGATGGAGGACCTGATCTGCCCGGTCGAATGG
95     D  G  V  I  L  H  H  I  L  A  P  L  R  P  D  L  F  F  Y  A  N  R  D  I  L  R  V  L  P  Q  M  E  D  L  I  C  P  V  E  W

721   CGGCAGGAGAAGCGGAGCCTGCAGAAGACGCGCGAGACGATGGACTACACCCGCCGCGCCATCGAGGCCGGGCGGCTGGGCGTAATCTTCCCCTCCGGGCGGCTGGCCAAGCGGACGGGA
136    R  Q  E  K  R  S  L  Q  K  T  R  E  T  M  D  Y  T  R  R  A  I  E  A  G  R  L  G  V  I  F  P  S  G  R  L  A  K  R  T  G

841   CTGAAGCTCATGGAGCGGCCGTGGATGGCCTCCGCTGCGATGATCGCGCGGAAGTTCGACCTGCCTGTGGCGCCGGTGAACATCCGGGCGCGCAACTCGACGCTGTTCTACCTCTTCGAC
176    L  K  L  M  E  R  P  W  M  A  S  A  A  M  I  A  R  K  F  D  L  P  V  A  P  V  N  I  R  A  R  N  S  T  L  F  Y  L  F  D

961   CTGATCCACCCCACGCTGCGGGACATCACTCTGTTCCACGAGGTGCTGAACAAGCATCGCCAGCCGTTCCGACTGACGATGGGCCGGCCGATCCCGGCCGAGCGTCTGCCGTCGAAGTCC
216    L  I  H  P  T  L  R  D  I  T  L  F  H  E  V  L  N  K  H  R  Q  P  F  R  L  T  M  G  R  P  I  P  A  E  R  L  P  S  K  S
                                                                                                                                S2
1081  GAAGAGGGGATCGAGATGCTGCGCGAGGCGACGCTCGCGCTCGGCGGGCAATATTCGCCTGCCGTCAGCATCCTGCAGATGACGAGCCTGCGCGGCGCGCTCCGCTACGCACGCGGGTAG
256    E  E  G  I  E  M  L  R  E  A  T  L  A  L  G  G  Q  Y  S  P  A  V  S  I  L  Q  M  T  S  L  R  G  A  L  R  Y  A  R  G
       BamHI
1201  GGGATCCCGGAGCGCGCGGCTGGCCGGACAGGCGGCCGCGCCTCCTCCCCACCCCTCCGCGGCCGGCAGGAGCTGTCGCCGCTGCGTTCCGGCAGCCGCGGGCGGCCATTTTCCTCATGC

1321  TCCTGCAGTGACCGGAGGACCCGTCGCCGCCGCGTCCAACGGCAGGCGCAGGACGCCCCCTTCGTCATGCTCTTGCACGGCGGCGCGGGATGGCGTCAACTCGCGCCATGACCGGACCGC

1441  GCTACACCCCGCTCGTCGAGAGCCTGCCCTCCACCGTTCCCTTCGTCGGCCCCGAGACGCAGGAGCGTGCGCGTGGCCGCCCGTTCCGCGCCCGCTTGGGCGCGAACGAGAGCCTCTTCG

1561  GCCCCTCGCCGCACGCCGTGGCAGCCATGGCCGCGGCCGCCGCCGAGGTCTCGCTGTATGGCGATCCAGAGATCCATGCGCTGCGCACTGCCATCGCGGCCCATCACGGCGTGGCGCCGG

1681  ATCAGGTGACGGTGGGCGAGGGCGTCGACGGGCTTCTGGGCAACCTCGTCCGGCTGCTTGTCGACGAGGGCGCGCCGGTCGTGACCTCGGAGGGCGCCTATCCGACCTTCGCCTTCCATG

1801  TGGCGGGGTTCGGCGGCCGCCTGCACAAGGTGCCCTACCAGGGCGATCACGAGGATCCCGAGGCGCTGGCGGCCGAGGCCCGGAGGTCGGGGCCCGGATCGTCTATTTCGCCAATCCGGA
                                                                                                          StuI
1921  CAATCCGATGGGGTCGCACCATCCTGCCGCCTCCGTCCGCGCCCTGATCGAGGCGCTGCCCGAGGGCACGCTGCTCGCGCTCGACGAGGCCTATGTGGAACTGGCCCCCGAGGGCACGGC

2041  GCCCGAGATCGCCCCCGAGGATCC
```

FIG. 6. DNA sequence of pCHB17B20. Only the proposed coding strand for the *sqdA* gene is shown. The protein sequence predicted by translation of the proposed *sqdA* coding sequence is given below the gene sequence. The locations of stop codons ($S^1$ and $S^2$) in frame with the coding sequence, a potential ribosome-binding site (SD), four possible initiation codons ($I^1$ to $I^4$), and restriction sites used to construct deletion clones are above the sequence and are underlined.

SWISS-PROT release 15) for homologous proteins of known function. However, no known protein showed significant homology to the putative *sqdA* protein.

## DISCUSSION

Our objective was to develop an experimental system which would facilitate a solution to the classical problem of the pathway of sulfolipid biosynthesis. A genetic approach to dissect the components of sulfolipid biosynthesis had never been tried before. The organism of choice for such a study is the purple nonsulfur bacterium *R. sphaeroides*, because it contains the plant sulfolipid, common genetic techniques are available for this bacterium, and this organism is able to grow in the absence of photosynthesis. Although the function of sulfolipid is not established, we were concerned that obligately photoautotrophic organisms, such as higher plants or cyanobacteria, would not be able to grow in the absence of sulfolipid, causing lethality of possible sulfolipid-deficient mutants.

By using a direct screening procedure, the examination of lipid extracts from chemically mutagenized *R. sphaeroides* cells by TLC, a series of mutants deficient in sulfolipid biosynthesis was isolated. In order to determine how many genes were altered in the collection of mutants and in order to facilitate the construction of isogeneic mutant and wild-type lines, we initially isolated and characterized a gene which complemented one of the mutants, *sqdA*.

Sequence analysis of the clone complementing the *sqdA* mutant revealed an ORF which could encode a 33.6-kDa protein. The analysis of the *sqdA* ORF was facilitated by the construction of the expression vector pCHB500 for *R. sphaeroides*, which allowed the orientation-dependent expression of ORFs to determine the coding strand for the *sqdA* gene.

The role of the *sqdA* gene product in sulfolipid biosynthesis could not be deduced on the basis of sequence homology of the translated protein sequence with protein sequences of known enzymes or any deduced amino acid sequence in the protein data banks NBRF-PIR (release 26) and SWISS PROT (release 15). It is therefore suggested that the *sqdA* gene product represents a novel enzyme involved in sulfolipid biosynthesis. Further experiments will be required to elucidate the function of the *sqdA* gene product. A preliminary search for precursors of sulfolipid biosynthesis among the sulfur-containing water-soluble compounds accumulating in the *sqdA* mutant did not reveal any reproducibly obtained novel compounds. Thus, we have not yet been able to identify a precursor of sulfolipid biosynthesis accumulating in the mutant.

The results presented here may be regarded as the initial steps in the development of *R. sphaeroides* as a model

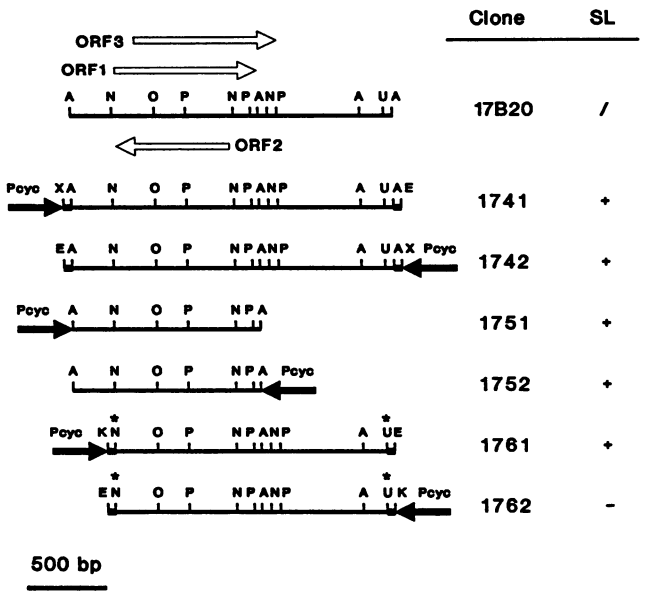


FIG. 7. Complementation analysis using derivatives of pCHB17B20 cloned into the expression vector pCHB500. The 2.1-kb *Bam*HI insert of pCHB17B20 is shown at the top; the orientation and location of three ORFs (ORF1, ORF2, and ORF3) are indicated by open arrows. For each clone shown, three independent constructs were prepared, which gave identical results in complementation assays. SL, presence (+) or absence (−) of the sulfolipid in lipid extracts of the sulfolipid-deficient *sqdA* mutant containing the indicated clone; pCHB17B20 (17B20) cannot be tested (/). Pcyc, *cycA* promoter (solid arrows). The direction of transcription is indicated by the arrowhead. Small stretches of pBluescript polylinker region flanking the inserts are indicated by a thicker line. Restriction sites: A, *Bam*HI; K, *Kpn*I; N, *Nae*I; O, *Xho*I; P, *Pst*I; U, *Stu*I; X, *Xba*I. Locations of *Nae*I and *Stu*I sites, no longer cleavable because of the cloning process, are indicated (*).

system for studying sulfolipid biosynthesis and function. We have demonstrated that sulfolipid-deficient mutants can be isolated and that the affected genes can be cloned by complementation. We anticipate that further exploration of the *R. sphaeroides* system may allow elucidation of the pathway for sulfolipid biosynthesis and will provide insights into the potentially novel biochemistry involved in this process.

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