Two enzymes of diacylglyceryl-*O*-4′-(*N,N,N,*-trimethyl)-homoserine biosynthesis are encoded by *btaA* and *btaB* in the purple bacterium *Rhodobacter sphaeroides*

Rouven M. Klug and Christoph Benning*

Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824-1319

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Betaine lipids are ether-linked, nonphosphorous glycerolipids that resemble the more commonly known phosphatidylcholine in overall structure. Betaine lipids are abundant in many eukaryotes such as nonseed plants, algae, fungi, and amoeba. Some of these organisms are entirely devoid of phosphatidylcholine and, instead, contain a betaine lipid such as diacylglyceryl-*O*-4′-(*N,N,N,*-trimethyl)homoserine. Recently, this lipid also was discovered in the photosynthetic purple bacterium *Rhodobacter sphaeroides* where it seems to replace phosphatidylcholine under phosphate-limiting growth conditions. This discovery provided the opportunity to study the biosynthesis of betaine lipids in a bacterial model system. Mutants of *R. sphaeroides* deficient in the biosynthesis of the betaine lipid were isolated, and two genes essential for this process, *btaA* and *btaB*, were identified. It is proposed that *btaA* encodes an *S*-adenosylmethionine:diacylglycerol 3-amino-3-carboxypropyl transferase and *btaB* an *S*-adenosylmethionine-dependent *N*-methyltransferase. Both enzymatic activities can account for all reactions of betaine lipid head group biosynthesis. Because the equivalent reactions have been proposed for different eukaryotes, it seems likely that orthologs of *btaA*/*btaB* may be present in other betaine lipid-containing organisms.

Polar lipids are essential components of all biological membranes. Most common are glycerolipids containing a diacylglycerol moiety to which a polar head group is attached. A head group can be a carbohydrate moiety as in the very abundant plant galactolipids or a phosphorylester as in the glycerophospholipids, the most common lipid class in animals. Betaine lipids represent a third class of glycerolipids in which a quaternary amine alcohol is bound in an ether linkage to the diacylglycerol moiety (1, 2). The overall structure of betaine lipids (Fig. 1) resembles to some extent that of the glycerophospholipid phosphatidylcholine (PC). Although the phase transition temperature for betaine lipid was found to be slightly higher compared with PC with identical fatty acid composition, the physical phase behavior of betaine lipid in mixtures with water is similar to that of PC (3). The betaine lipid diacylglyceryl-*O*-4′-(*N,N,N,*-trimethyl)homoserine (DGTS) (4) and a closely related isoform diacylglyceryl-*O*-2′-(hydroxymethyl)(*N,N,N*-trimethyl)-β-alanine were discovered in the unicellular alga (Chrysophyceae) *Ochromonas danica*. Soon after, it became apparent that betaine lipids are abundant and widespread in nonseed plants and alga (5–13). Furthermore, they are common in fungi including edible mushrooms and human pathogens (14–16) and are also present in amoebae (17). Although betaine lipids are abundant in primitive vascular plants such as ferns, they are absent from all tested seed plants (11, 14). Recently, the betaine lipid DGTS also has been discovered in the photosynthetic purple bacterium *Rhodobacter sphaeroides* (18) and the plant-nodule-forming bacterium *Sinorhizobium meliloti* (19). It has been pointed out repeatedly (2, 14) that there is a peculiar inverse relationship between the presence of betaine lipids and PC in different organisms, indicating that betaine lipids may substitute for PC. This hypothesis is supported by the accumulation of DGTS in the two bacteria mentioned above only when these are grown under phosphate-limiting conditions, under which the relative amounts of glycerophospholipids including PC are decreased. In *S. meliloti* the regulator encoded by *phoB* has been implicated in the control of DGTS biosynthesis after phosphate deprivation (19).

Feeding experiments with methionine labeled at specific carbon atoms in algae and moss consistently suggest that *S*-adenosylmethionine (SAM) donates the 3-amino-3-carboxypropyl portion of the DGTS head group as well as the three methyl groups giving rise to the quaternary ammonium (20–23). Because similar results were obtained with *R. sphaeroides* (24), it seems plausible that the pathway for DGTS biosynthesis is universal in eukaryotes and prokaryotes. For this reason we chose *R. sphaeroides* as the initial model organism to identify the genes and enzymes essential for DGTS biosynthesis. A similar approach was successfully used to clone the first genes essential for the biosynthesis of the sulfolipid sulfoquinovosyldiacylglycerol from *R. sphaeroides* (25, 26) and subsequently the orthologs from cyanobacteria and the plant *Arabidopsis thaliana* based on sequence similarity to the genes from *R. sphaeroides* (27, 28).

Materials and Methods

Strains, Plasmids, Media, and Growth Conditions. Bacterial strains and plasmids (26, 29–32) are shown in Table 1. Cells of *R. sphaeroides* were grown photoheterotrophically in modified Sistrom's medium (33, 34) containing 50 mM Hepes-KOH, pH 6.8, and different potassium phosphate concentrations as described (18, 26). If required 25 μg/ml kanamycin, 100 μg/ml rifampicin, or 0.8 μg/ml tetracycline was added. Strains of *Escherichia coli* were grown in Luria broth. Antibiotics were added at concentrations of 100 μg/ml ampicillin, 50 μg/ml kanamycin, or 10 μg/ml tetracycline.

Mutant Isolation Procedure and Complementation Assay. Cells of *R. sphaeroides* wild type were mutagenized with *N*-methyl-N′-nitro-*N*-nitrosoguanidine (26). Colonies were grown under phosphate-limiting conditions and screened for polar lipid mutants by analyzing lipid extracts on high-performance TLC plates (HPTLC 60, Merck) essentially as described (26). However,

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Abbreviations: PC, phosphatidylcholine; DGHS, diacylglyceryl-*O*-4′-homoserine; DGMS, diacylglyceryl-*O*-4′-(*N*-monomethyl)homoserine; DGTS, diacylglyceryl-*O*-4′-(*N,N,N,*-trimethyl)homoserine; SAM, *S*-adenosylmethionine.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF329857).

*To whom reprint requests should be addressed. E-mail: benning@msu.edu.

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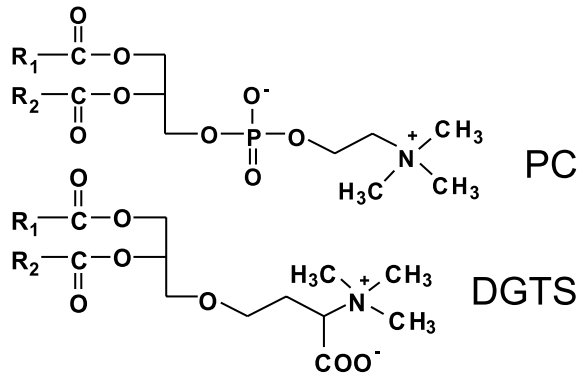

Fig. 1. Structures of PC and DGTS. R_1 and R_2 represent the hydrocarbon chains of the respective acyl groups.

polyhydroxybutyrate, which accumulates in phosphate-stressed cells, was removed from the extracts by precipitation after the addition of 4 vol of hexane. The TLC plates were developed with chloroform, acetone, methanol, acetic acid, and water (50:20:10:10:5 by volume), and the lipids were visualized with iodine vapor. Cosmids and other transferable clones were conjugated into the mutant RKL3 by triparental mating as described (26), and exconjugants were tested for complementation by analyzing lipid extracts as mentioned above.

Quantitative Lipid Analysis. Cultures (50 ml) of mutant or wild-type strains were grown photoheterotrophically under phosphate-limiting conditions to late logarithmic phase. Cells were harvested by centrifugation, and lipid extracts were prepared as described (35). Lipids were separated by two-dimensional TLC (18), and fatty methyl esters were prepared from each lipid and quantified by gas chromatography as described (26). From these data the mol % fraction of the analyzed polar lipids was calculated for each lipid.

DNA Manipulations. Standard DNA manipulations were performed according to Sambrook *et al.* (36) or as suggested by manufacturers of enzymes and kits. The complete insert of pRKL323 was cloned into pBluescript II SK(+) (Stratagene) and sequenced on both strands by the Michigan State University Sequencing Facility. Database searches were conducted at the web site of the National Center for Biological Information (http://www.ncbi.nlm.nih.gov/) using BLAST algorithms (37). For the construction of p*btaA* and p*btaA*NT, *btaA* and *btaA*NT (*bta* N-terminally Truncated) were amplified from pRKL323 by standard PCR using the oligonucleotides 5′-CTTCTAGACGAGGCGAGGCAACGACAGG-3′ and 5′-CTTCTAGACGAAGGACTGATGGAGCGGATGT-3′ as forward primers, respectively, containing an *Xba*I site and 5′-TCGAATTCGGTAGGTCGCGTCCATCAGC-3′ as reverse primer containing an *Eco*RI site. The PCR products were digested with *Xba*I and *Eco*RI and inserted into the corresponding restriction sites of pCHB500. The *btaB* inactivation cassette of p*btaB*-dis (*btaB* disrupted) was constructed by deleting the bases 245–397 of *btaB* and inserting the neomycin phosphotransferase gene from pUC4K. For this purpose, *btaB* was amplified by PCR using 5′-GCGAATTCTACGGCGGCTTCCACCTCTACC-3′ and 5′-GAGAATTCCTCTGCAACACCGGCTCCACACC-3′ as primer pair and cloned into the *Eco*RI site of pBluescript II SK(+), giving rise to plasmid p*btaB*. The deletion in *btaB* was introduced by inverse PCR of the whole plasmid using the following primer set: 5′-ATAGATCTAGGCGGCGCTTCATCTCGTG-3′ and 5′-ATAGATCTAGGCCAGCATCTCCTGCGAG-3′, both containing a *Bgl*II site. The construction of the inactivation cassette was completed by ligating the 1.3-kb *Bam*HI neomycin phosphotransferase fragment of pUC4k with the PCR product cut with *Bgl*II. The complete inactivation cassette was cut with *Eco*RI and cloned into the corresponding restriction site of pSUP202. The resulting plasmid p*btaB*-dis was used to inactivate the respective wild-type gene in *R. sphaeroides* wild-type cells as described (35). For Southern analysis, probes were labeled by random priming using a kit from Amersham Pharmacia. DNA fragments were separated on agarose gels blotted onto Hybond-N^+ nylon membranes (Amersham Pharmacia) by the alkaline transfer method according to the manufacturer's instructions. The DNA was hybridized according to the method of Reed and Mann (38).

Structural Elucidation. Fast atom bombardment mass spectrometry measurements were done at the Michigan State University Mass Spectrometry Facility. Standard ^{1}H-NMR spectra were

Table 1. Description of strains and plasmids used in this study

Strain or plasmid	Description or construction	Source or ref. no.
R. sp. 2.4.1	Wild type	ATCC 17023
R. sp. RKL3	DGTS-deficient *btaA* MNNG-induced mutant	This study
R. sp. btaB-dis	DGTS-deficient *btaB* disruption mutant	This study
E. coli HB101	F^- Δ(*mcrC-mrr*) *leu supE44 ara14 galK2 lacY proA2 rpsL20* (Str^r) *xyl-5 mtl-1 recA13*	30
E. coli XL-10 Gold	Tet^rΔ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*) 173 endA1 *supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacIqZDM15* Tn*10* (Tet^r) Amy Cam^r]	Stratagene
E. coli DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ*80dlacZDM15* Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galKl-rpsL nupG*	GIBCO/BRL
E. coli MM294	F^- *endA1 hsdR17* ($r_k^-m_k^-$) *supE44 thi-1 relA1*	29
pBluescript II SK(+)	Amp^r	Stratagene
pRK2013	Kan^r Tra^+ RK2-ColE1$_{rep}$	31
pCHB500	Tc^r; expression vector for *R. sphaeroides*	26
pUC4K	Kan^r Nm^r; contains neomycin phosphotransferase gene of Tn903	Pharmacia
pSUP202	Ampr Cmr Tcr pBR325rep	32
pRKL301	Cosmid clone complementing RKL3	This study
pRKL323	Smallest subclone of pRKL301 complementing RKL3	This study
p*btaA*	Nucleotides 436–1834 of pRKL323 in pCHB500	This study
p*btaA*NT	Nucleotides 633–1834 of pRKL323 in pCHB500	This study
p*btaB*	Nucleotides 1814–2625 of pRKL323 in pBS II SK(+)	This study
p*btaB*-dis	*btaB* inactivation cassette in pSUP202	This study

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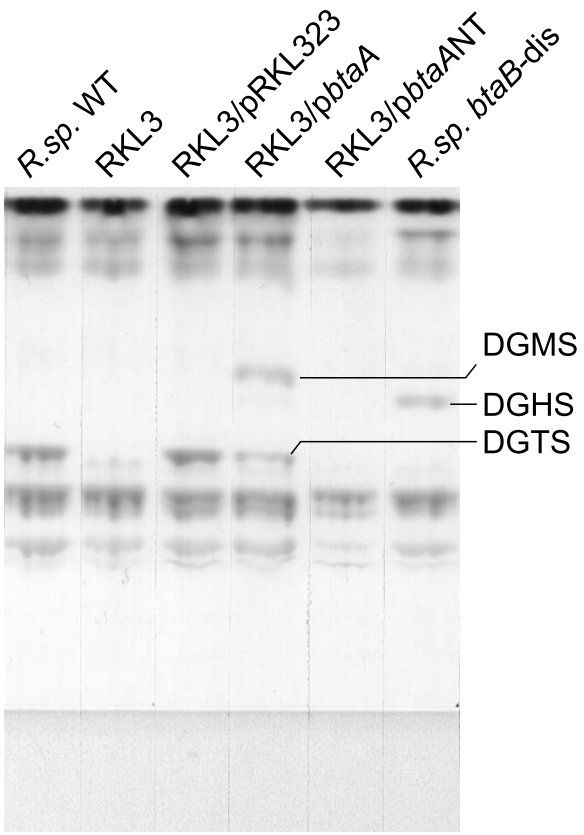

Fig. 2. Comparison of lipid extracts from different strains of *R. sphaeroides*. All cells were grown under phosphate-limited conditions at an initial P_i concentration of 0.1 mM. A one-dimensional thin-layer chromatogram stained by iodine vapor is shown. The indicated strains and plasmids are described in Table 1 and in the text.

recorded with a Varian VXR500 spectrometer (500 MHz for protons) at 25°C using CD3OD/CDCl3 (1:1, vol/vol) as solvent. For each spectrum 200 acquisitions at a recycle delay of 0.2 s were measured. The signal of the deuterated methanol at 3.30 ppm was used as internal standard.

Results

Isolation of DGTS-Deficient Mutants. To isolate genes essential for betaine lipid biosynthesis we used a genetic approach. Based on our previous experience with the isolation of sulfolipid- and phosphatidylcholine-deficient mutants of *R. sphaeroides* (35, 39) and the fact that DGTS is only conditionally present in this bacterium, we rationalized that the loss of DGTS may not be lethal and, therefore, the respective mutant could be readily isolated. To enhance the mutation frequency, we treated *R. sphaeroides* with a mutagenic compound such that the fraction of surviving cells was 1–2% and the occurrence of rifampicin-resistant colonies was increased more than 10-fold. Mutagenized cells were plated on Sistrom's medium that contained 0.1 mM phosphate, leading to phosphate deprivation, a condition inducing the synthesis of DGTS in *R. sphaeroides* (18). Screening only 288 clones of this mutagenized population by extraction of lipids from individual colonies and analysis of lipid extracts by one-dimensional TLC, six putative mutants with altered lipid composition were identified. Among these was one, RKL3, that apparently lacked DGTS. A thin-layer chromatogram developed under the same conditions as used for screening is shown in Fig. 2, depicting lipid extracts of the wild-type and the RKL3 mutant lines (Fig. 2, left two lanes).

Table 2. Lipid composition of *R. sphaeroides* wild type and RKL3 after phosphate deprivation

Lipid	Wild type, mol %	RKL3, mol %
MHDG	1.3 ± 0.2	12.7 ± 1.6
DGTS	15.9 ± 1.7	n.d.
MPE	n.d.	0.9 ± 0.2
GGDG	32.6 ± 2.4	36.7 ± 2.8
PE	1.5 ± 0.1	7.5 ± 0.4
OL	21.2 ± 3.6	15.2 ± 0.1
PG	4.7 ± 0.6	7.5 ± 0.4
SQDG	17.5 ± 0.9	14.5 ± 0.7
PC	0.1 ± 0.1	1.1 ± 0.6
PL	5.3 ± 0.5	3.7 ± 0.2

Mean values from three independent cultures (0.1 mM P_i) and standard errors are shown. GGDG, glucosylgalactosyldiacylglycerol; MHGD, monohexosyldiacylglycerol; MPE, *N*-monomethylphosphatidylethanolamine; n.d., not detected; OL, ornithine lipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, undefined phospholipid; SQDG, sulfoquinovosyldiacylglycerol.

Mutant Line RKL3 Lacks the Betaine Lipid DGTS. To reveal the full complexity of the mutant lipid spectrum, lipids from phosphate-deprived cultures of wild type and RKL3 were compared by using a two-dimensional chromatography system. The spot for DGTS was clearly missing from the RKL3 chromatogram. Instead, the spot for phosphatidylethanolamine was more pronounced and a new faint spot appeared. Because we initially suspected that the new compound present in the RKL3 might be related to the defect in DGTS biosynthesis, we isolated this lipid and subjected it to ^{1}H-NMR analysis. The ^{1}H-NMR spectrum of this lipid (data not shown) was in agreement with a spectrum published for monomethylphosphatidylethanolamine (40). Furthermore, the exact mass determination by fast atom bombardment mass spectrometry provided a value of 758.5716 m/z, very close to the predicted mass for this compound (758.5700 m/z) containing two vaccenic acyl groups (18:1 *cis* Δ^{11}). We observed this lipid on occasion in wild-type extracts and also noticed it to be absent in more rare cases from RKL3 extracts. Therefore, the accumulation of monomethylphosphatidylethanolamine was not related to a deficiency in DGTS biosynthesis and was affected by the culturing conditions in ways we did not further explore. Nevertheless, we included monomethylphosphatidylethanolamine in the quantitative analysis as shown in Table 2. No traces of DGTS were detectable in the extract of the RKL3 mutant strain, which also showed an increase in relative amounts of the two glycolipids monohexosyldiacylglycerol and glucosylgalactosyldiacylglycerol (Table 2). Furthermore, phospholipids were not as strongly reduced in the RKL3 mutant compared with wild type under the used phosphate-limited growth conditions. Taken together, these data identified RKL3 as a DGTS-deficient mutant of *R. sphaeroides.*

DGTS Biosynthesis Is Restored by a Wild-Type DNA Fragment Containing a Two-ORF Operon. The isolation of the DGTS-deficient RKL3 mutant provided the basis for the cloning of the corresponding wild-type gene by genetic complementation. For this purpose, individual cosmids containing 20- to 30-kb fragments of wild-type DNA (26) were transferred by conjugation into the RKL3 mutant line. Exconjugants were analyzed for restoration of DGTS biosynthesis by TLC analysis of lipid extracts as in the original mutant screen. Examining 150 clones, one cosmid was identified, RKL301, that rescued the mutant phenotype. A small subclone library was prepared by partial digestion of this cosmid in the expression vector pCHB500, and the subclones were tested again for their ability to restore the DGTS defect in RKL3. Among 384 subclones, 13 complementing clones with inserts of variable length were identified and seven were further characterized (Fig. 3*A*). The complementing clone, pRKL323 (Fig. 2,

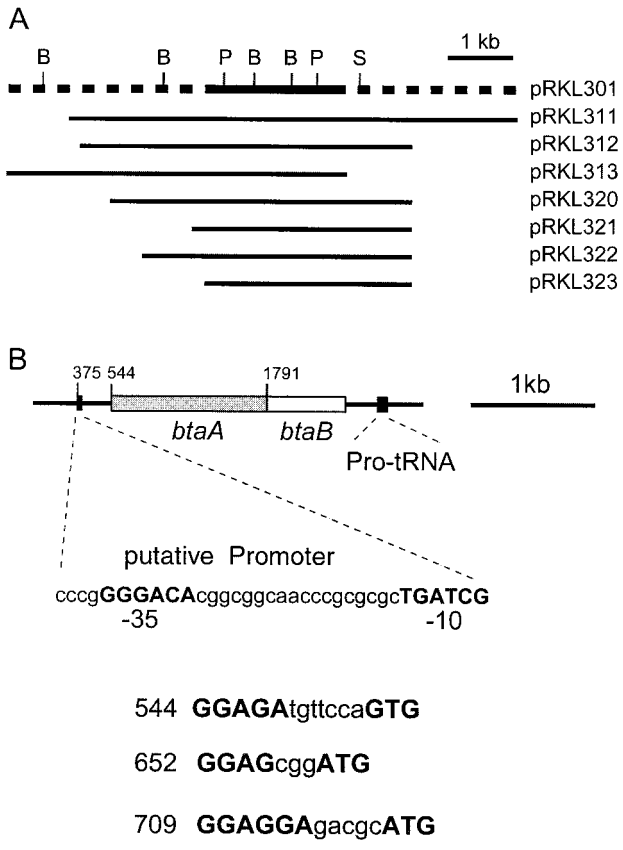

Fig. 3. Cloning and analysis of the DGTS operon of *R. sphaeroides*. (*A*) Partial restriction map of cosmid pRKL301 complementing RKL3 and complementing subclones of pRKL301. The region of pRKL301 common to all subclones is indicated by solid line. B, *Bam*HI; P, *Pst*I; S. *Sal*I. (*B*) Structure of the insert of pRKL323 derived from sequence analysis. The two ORFs associated with DGTS biosynthesis, *btaA* and *btaB*, are drawn as gray and open boxes, respectively. In addition, a putative promoter (filled box) with consensus sequences (bold) and a putative proline-tRNA gene (Pro-tRNA) are indicated. Three potential start codons (bold) and ribosome binding sites (bold) are shown. Numbers refer to nucleotides of the sequence deposited at GenBank (accession no. AF329857).

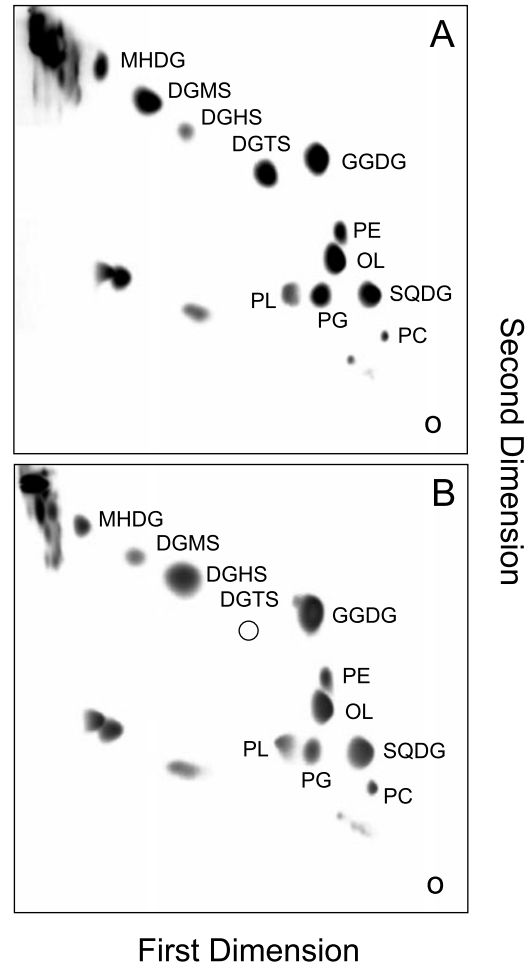

Fig. 4. Lipid phenotype of RKL3 containing p*btaA* (*A*) and a mutant disrupted in *btaB* (*B*). The cells were grown under phosphate-limited conditions at an initial P_i concentration of 0.1 mM. Abbreviations are as defined in the footnote and the legend to Table 2.

lane 3), with the smallest insert of ≈3 kb (Fig. 3*A*) was submitted to sequence analysis. Close examination of the DNA sequence revealed two adjacent ORFs overlapping by 2 bp that were tentatively designated *btaA* and *btaB* (Fig. 3*B*). Furthermore, a putative promoter with −35 and −10 consensus sequences was identified upstream of the *btaA* ORF (Fig. 3*B*). Downstream of the *btaB* gene a putative Pro-tRNA gene was located. Taken together, these sequence features of the complementing DNA fragment suggested that it contains a small two-ORF operon involved in DGTS biosynthesis.

Expression of *btaA* in RKL3 Restores DGTS Biosynthesis and Leads to the Accumulation of Diacylglyceryl-*O*-4′-Homoserine (DGHS). Three potential initiation codons with upstream ribosome binding site consensus sequences were present at the 5′ end of the putative *btaA* ORF (Fig. 3*B*). To determine whether *btaA* or *btaB* was affected in RKL3 and to examine which of the potential initiation codons of *btaA* was actually used, a long version of the *btaA* ORF containing the furthest upstream initiation codon (Fig. 3*B*, nucleotide 544) as well as a shorter version containing only the second and third initiation codons (Fig. 3*B*, nucleotides 652 and 709) were inserted into the expression vector pCHB500 under the control of the cytochrome *c* promoter, which is not affected by phosphate availability. The introduction of the construct containing the longer ORF (p*btaA*) into RKL3 resulted in restoration of DGTS biosynthesis whereas the truncated construct (p*btaA*NT) failed to rescue the mutant phenotype (Fig. 2, lanes 4 and 5). Therefore, *btaA* starting at the first possible initiation codon (Fig. 3*B*, nucleotide 544) encodes a protein essential for DGTS biosynthesis in *R. sphaeroides.* It is also the gene mutated in RKL3. Upon closer examination of lipid extracts from RKL3 harboring the plasmid p*btaA*, we discovered the appearance of two other lipid spots [Fig. 4*A*, diacylglyceryl-*O*-4′-(*N*-monomethyl)homoserine (DGMS) and DGHS]. The material from both spots was isolated and analyzed by ^{1}H-NMR and mass spectrometry. One spot (Fig. 4, DGMS) represented a mixture of divaccenic acylglyceryl-*N*-monomethylhomoserine (736.6058 *m/z*; expected 736.6091 *m/z*) and divaccenic acylglyceryl-*N*-dimethylhomoserine (750.6248 *m/z*, expected 750.6248 *m/z*), and the other divaccenic acylglycerylhomoserine (DGHS; 722.5923 *m/z*, expected 722.5934 *m/z*). Chemical shift (coupling constants) assignments in ppm (Hz) for the ^{1}H-NMR spectrum of DGHS were as follows: Homoserine H-2, 3.58,($J_{1,2a}$ 7.0, $J_{1,2b}$ 3.5); H-3a, 2.14; H-3b, 2.03; H-4, 3.65. Glycerol H-1a, 4.33 ($J_{1a,1b}$ 12.2, $J_{1a,2}$ 3.5); H-1b, 4.11 ($J_{1b,2}$ 6.5); H-2, 5.2 ($J_{2,3}$ 5.6); H-3, 3.55. Fatty acids -CH_3- 0.8–0.9; -CH_2- 1.2–1.4; -βCH_2, 2.29–2.31; -βCH_2, 1.59; =CH-5.3; -CH_2-CH = 1.99. Growing of RKL3 harboring p*btaA* under phosphate- sufficient condition

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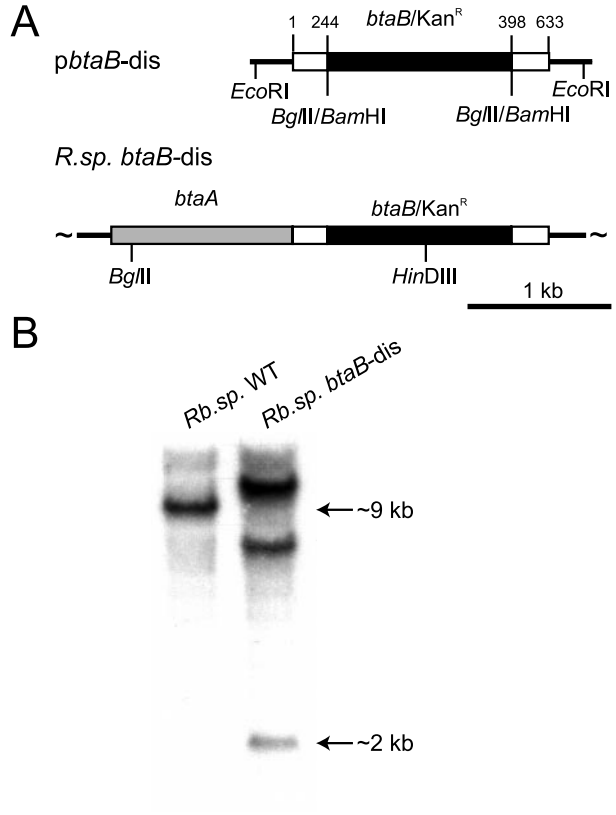

Fig. 5. Disruption of the *btaB* gene. (*A*) Maps of the inactivation construct p*btaB*-dis and the genomic DNA surrounding the inactivated *btaB* gene in *R. sphaeroides btaB*-dis. The kanamycine resistance cassette is drawn as a black box. Numbers refer to nucleotides of the *btaB* ORF (open box). (*B*) Genomic DNA blot of *R. sphaeroides* and *btaB*-dis strain probed with the *btaB* wild-type gene. Genomic DNA was digested with *Bgl*II and *Hin*DIII. The blot was probed with the insert of p*btaB*. Diagnostic hybridizing fragments visualized by exposure to x-ray film and their approximate length are indicated.

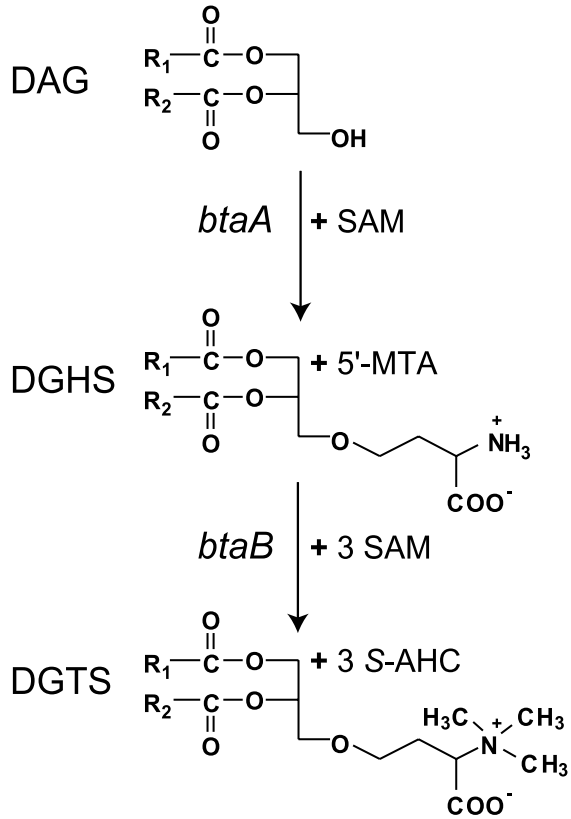

Fig. 6. Hypothesis for the function of *btaA* and *btaB* in DGTS biosynthesis in *R. sphaeroides.* DAG, diacylglyceryl; 5′-MTA, 5′-methylthioadenosine; *S*-AHC, *S*-adenosylhomocysteine.

led to the formation of small amounts of DGHS but not DGTS (data not shown). Taken together, these observations suggested that *btaA* may be involved in the formation of DGHS, a postulated precursor of DGTS biosynthesis (24).

Inactivation of *btaB* Causes DGTS Deficiency and Accumulation of DGHS Under Low-Phosphate Growth Conditions. The predicted amino acid sequence of *btaA* revealed only weak similarity to a bacterial methyltransferase and no further clues toward its possible function after database searches. However, the predicted amino acid sequence of the second ORF in the operon, *btaB*, showed high similarity to methyltransferases and contained clear protein motifs characteristic for SAM binding sites of methyltransferases (41, 42). This observation suggested that *btaB* encodes an SAM-dependent *N*-methyltransferase postulated to be involved in DGTS biosynthesis in *R. sphaeroides* (24). To test this hypothesis, we inactivated *btaB* in *R. sphaeorides* wild type by replacing an internal fragment with a kanamycin resistance cassette (Fig. 5*A*). For confirmation, Southern analysis was conducted by digesting genomic DNA with *Bgl*II/*Hin*DIII and probing with the insert of p*btaB*. The radiogram of the *btaB* disruption line, *R. sp. btaB*-dis, indicated that a ≈9-kb fragment diagnostic for the wild-type chromosome was completely missing (Fig. 5*B*) in agreement with a loss of all wild-type copies of *btaB*. Instead, a ≈2-kb fragment predicted for the disrupted *btaB* gene was present in *R. sp. btaB*-dis. A second fragment, for which the size could not be predicted due to the lack of genomic sequence information, was expected for *R. sp. btaB*-dis in this experiment. However, the unexpected third fragment (Fig. 5*B*) may have been the result of incomplete digestion. Analysis of the lipid extract from *R. sp. btaB*-dis revealed complete DGTS deficiency (Figs. 2 and 4*B*). Therefore, *btaB* as shown for *btaA* above, is essential for DGTS biosynthesis in *R. sphaeroides*. Moreover, the closer examination of the lipid extract from *R. sp. btaB*-dis grown under phosphate limitation revealed the accumulation of DGHS (Fig. 4*B*, DGHS) and to some extent of partially methylated DGHS (Fig. 4*B*, DGMS).

Discussion

During evolution, living organisms adopted a wide range of amphipathic molecules that can serve as polar lipids. Membranes of some bacteria, such as *R. sphaeroides,* contain a rich complement of polar lipids. Its biochemical resourcefulness allows *R. sphaeroides* not only to change the membrane lipid composition under adverse conditions, e.g., phosphate starvation (18, 35); it also has the capability to incorporate xenobiotic compounds such as the buffer substance Tris(hydroxymethyl)aminomethane (Tris) to form phosphatidyl-Tris (43). Because *R. sphaeroides* genetics is straightforward, this bacterium represents a very useful model organism for the identification of genes that encode enzymes essential for the biosynthesis of some of the less well-known membrane lipids that are, nevertheless, very common in large groups of organisms.

Screening only a few hundred heavily mutagenized colonies of *R. sphaeroides,* we were able to identify a mutant, RKL3, unable to produce DGTS when grown on low-phosphate medium. Brute force complementation cloning using RKL3 yielded a wild-type DNA fragment containing a putative two-gene operon. Expression of the first gene, *btaA*, under the control of the *Rhodobacter*

capsulatus cytochrome *c* promoter of pCHB500 (26) in RKL3 restored DGTS biosynthesis and led to the accumulation of DGHS as well as partially methylated DGHS under low-phosphate growth conditions (Fig. 4*A*). Apparently, *btaA* is overexpressed in this strain and more DGHS is produced than can be processed by the DGHS methylase. The expression of *btaA* under the control of the cytochrome *c* promoter is not affected by phosphate deprivation. When RKL3 containing p*btaA* was grown on high-phosphate medium, no DGTS was formed and DGHS accumulated (data not shown), presumably because the phosphate-induced DGHS methylase activity was not present under these conditions. The database search using the predicted *btaA* gene product revealed only weak similarity to a bacterial methyltransferase and two other proteins of unknown function. Considering labeling data obtained by Hofmann and Eichenberger (24), which indicated that the homoserine moiety in DGTS is derived from SAM, the accumulation of the intermediate DGHS in RKL3/p*btaA* strongly suggests that *btaA* encodes an SAM:diacylglycerol 3-amino-3-carboxypropyl transferase catalyzing the first step of DGTS biosynthesis in *R. sphaeroides* (Fig. 6). Although rare, SAM-dependent 3-amino-3-carboxypropyl transferases are known to participate in other bacterial syntheses, e.g., in the biosynthesis of the antibiotic nocardicine (44).

The metabolite DGHS has been proposed to be the intermediate of DGTS biosynthesis in *R. sphaeroides* (24) that becomes *N*-methylated in an SAM-dependent reaction. Interestingly, the protein predicted to be encoded by *btaB* shows similarity to SAM-dependent methyltransferases. Two of the three characteristic amino acid sequence motifs (amino acids 47–55, motif I and amino acids 137–146, motif II) are clearly recognizable (41, 42), whereas motif II (amino acids 110–117) is less obvious. The strong accumulation of DGHS following the disruption of *btaB* in *R. sp. btaB*-dis grown on low-phosphate medium (Fig. 4*B*) is consistent with a defect in *N*-methylation leading to the accumulation of the DGTS precursor DGHS. However, assuming that *btaB* is completely inactivated in this line, another methyltransferase must be present under these conditions that is able to methylate DGHS with low efficiency, because a fairly small amount of partially methylated DGHS is present in the *R. sp. btaB*-dis mutant. Furthermore, this methyltransferase seems only capable of transferring one or two methyl groups to DGHS because DGTS is clearly not formed in the *btaB* disruption line. Therefore, it seems highly probable that *btaB* encodes the SAM:DGHS methyltransferase required for DGTS biosynthesis in *R. sphaeroides* (Fig. 6). The *pmtA* encoded SAM:phosphatidylethanolamine methyltransferase of *R. sphaeroides* catalyzes all three *N*-methylations during phosphatidylcholine biosynthesis (39). However, in yeast two distinct enzymes are involved in the sequential *N*-methylation of phosphatidylethanolamine (45). At the present time, we cannot fully exclude the possibility that the *btaB* gene product primarily catalyzes the later methylation steps during DGTS biosynthesis and that another methyltransferase catalyzes the first methylation reactions.

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