Two enzymes, BtaA and BtaB, are sufficient for betaine lipid biosynthesis in bacteria

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Abstract

Betaine lipids are non-phosphorous glycerolipid analogs of phosphatidylcholine. The biosynthesis of the betaine lipid diacylglyceryl-N,N,N-trimethylhomoserine has previously been studied in phosphate-starved cells of the purple bacterium Rhodobacter sphaeroides, and a genetic approach identified two proteins that are necessary for this process. Here, we show that all reactions of DGTS biosynthesis in R. sphaeroides are attributable to RsBtaA and RsBtaB, as co-expression of the respective genes leads to DGTS formation in Escherichia coli, which normally lacks this lipid. The recombinant RsBtaA protein was membrane-associated and showed S-adenosylmethionine/diacylglycerol 3-amino-3-carboxypropyl transferase activity. RsBtaA directed the transfer of label from 1-[14C]S-adenosylmethionine or [14C]diacylglycerol at equal rates into the betaine lipid precursor diacylglycerylhomoserine identifying both metabolites as the substrates of the reaction. Comparative analysis of RsBtaA and its bacterial orthologs revealed a motif with similarity to the AdoMet binding pocket of methyltransferases, and allowed the prediction of residues involved in substrate binding.

Keywords: Betaine lipid; Methyltransferase; Membrane biogenesis; Non-phosphorous lipid; One carbon metabolism; Phosphate starvation; SAM metabolism; DAG metabolism; Rhodobacter sphaeroides; Membrane associated enzyme

Many organisms, such as bacteria, plants, and fungi, rely on mineral nutrients taken directly from the soil or aquatic environment, and therefore tend to have exquisitely specialized mechanisms to cope with limitation of a given essential nutrient. For example, most organisms have well defined responses to phosphate limitation, including the replacement of cellular membrane phospholipids with non-phosphorous lipids. This last point has been well documented in the plants Arabidopsis [1,2] and oat [3], and most pronouncedly in the α-proteobacteria Rhodobacter sphaeroides and Sinorhizobium meliloti, which under phosphate stress become depleted of membrane phospholipids and induce the synthesis of non-phosphorous lipids, such as glycolipid species, the betaine lipid diacylglyceryl-N,N,N-trimethylhomoserine (DGTS), and ornithine containing lipids. [4,5].

DGTS, which was first discovered in the unicellular alga Ochromonas danica [6] and thereafter in Chlamydomonas reinhardtii [7], and other lower plants and fungi [8–10], has been proposed to take the place of phosphatidylcholine (PtdCho) in membranes of these organisms. Studies of DGTS biosynthesis using radioisotope feeding experiments identified methionine as a source of both the C₄ homoserine moiety and methyl groups of DGTS in Chlamydomonas [11]. These results led to the conclusion that S-adenosylmethionine (AdoMet) is the active donor of the four carbon unit of methionine leading to the synthesis of the intermediate diacylglycerylhomoserine (DGHS), and that DGTS arises from lipid-linked methylation of DGHS by an AdoMet-dependent methyltransferase. A similar pathway was

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proposed for *R. sphaeroides* based on labeling experiments [12] (Fig. 1).

Recently, Klug and Benning [13] used a genetic approach in *R. sphaeroides* to gain access to genes that are required for the biosynthesis of DGTS. This work resulted in the identification of an operon containing two open-reading frames, designated *btaA* and *btaB*, which appeared to be necessary for DGTS accumulation during phosphate deprivation in this bacterium. The product of *btaA* was proposed to function as an AdoMet/diacylglycerol 3-amino-3-carboxypropyl transferase, producing the intermediate DGHS. *Rs*BtaB showed a high degree of similarity to methyltransferases, and was proposed to be a trifunctional AdoMet-dependent *N*-methyltransferase, adding three methyl units to the amino group of DGHS to form DGTS (Fig. 1). In fact, *Rs*BtaB shows a high degree of sequence similarity to *Rs*PmtA, which produces phosphatidylethanolamine by *N*-trimethylation of phosphatidylethanolamine [14], indicating that *Rs*BtaB and *Rs*PmtA may share analogous functions in separate pathways. However, a biochemical analysis of the respective proteins has not been previously done. To study DGTS biosynthesis at the level of the enzymes and understand bacterial DGTS biosynthesis in greater detail, we initiated a biochemical characterization of the BtaA and BtaB proteins from *R. sphaeroides*, focusing in particular on *Rs*BtaA due to the unusual nature of the proposed reaction.

**Materials and methods**

**Materials**

Phospholipase C from *Bacillus cereus* was from Sigma (St. Louis, MO), mouse monoclonal anti-His tag antibody was from Qiagen (Valencia, CA, USA). Restriction enzymes, T4 DNA ligase, calf-intestinal phosphatase, and Klenow fragment were from New England Biolabs (Beverly, MA, USA), and *Taq* DNA polymerase was from Roche (Indianapolis, IN, USA). Labeled precursors were purchased from American Radiolabeled Chemicals, St. Louis, MO, USA. All other chemicals and solvents were of reagent grade and were from Sigma, EM Science (Gibbstown, NJ, USA) or J.T. Baker (Phillipsburg, NJ, USA).

**Expression of Rs*btaA* and Rs*btaB**

A summary of all strains and plasmids used in this study is presented in Table 1. The coding regions of *Rs*btaA and *Rs*btaB were PCR-amplified from plasmid pRK323 [13] for expression in pQE-31 (Qiagen) or pACYC-31 [15], respectively, with the following primers (restriction sites underlined): *btaA* forward (*Sph*I), 5′-ACATGCATGCAGTGACGCAGTTCGCCCTC-3′; *btaA* reverse (*Kpn*I), 5′-CGGGGTACCAGGACGATCCGCTCGAACCG-3′; *btaB* forward (*Bam*HI), 5′-GATGACCGACGCCACCCATGC-3′; *btaB* reverse (*Hin*dIII), 5′-GCAAGCTTCCTCTACCGTGGAGCGTG-3′. PCR was carried out with *Taq* DNA polymerase (Roche) according to the manufacturer’s specifications, except that 10% DMSO (v/v) was added to each reaction to overcome difficulties in PCR due to the high G+C content of *R. sphaeroides* DNA. PCR products were first cloned into PCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced at the MSU Genomics Technology Support Facility, followed by subcloning into the expression vectors using restriction sites as outlined in the PCR primers (see Table 1). The resulting constructs were thus designated pBtaA (*btaA* sequence in pQE-31), and pBtaB (*btaB* in pACYC-31).
PCR2.1 and pQE-31 derivatives were propagated in LB medium containing ampicillin at 100 μg/ml, and pACYC-31 derivatives in LB with chloramphenicol at 25 μg/ml. Strains containing compatible combinations of plasmids as indicated in individual experiments were constructed by co-transformation of heat-shock competent cells of TOP10F’ (Invitrogen) with 100 ng of both plasmids followed by selection on LB containing both ampicillin and chloramphenicol.

**In vivo production of DGHS and DGTS in *E. coli* and *R. sphaeroides***

*Escherichia coli* TOP10 F’ cells harboring compatible combinations of plasmids as described in individual experiments were grown as 2 ml overnight cultures, and 0.1 ml of these cultures were used to inoculate 10 ml LB containing appropriate antibiotics. After growing to OD₆₀₀ of 0.7–0.9, IPTG was added at 50 μM to induce low level expression of RsBtaA and to avoid the formation of inclusion bodies, and the culture was shifted to 28 °C and incubated further 4 h. Cells were harvested by centrifugation and suspended in 4 ml lysis buffer (20 mM KPi, pH 7.2, 500 mM NaCl, 15% (v/v) glycerol), frozen in liquid nitrogen and thawed slowly, and then lysed by sonication for 3-4 times, 30 s with a microprobe tip. The crude lysate was centrifuged at 20000 g for 10 min to remove unbroken cells and cellular debris, and the supernatant was subjected to centrifugation at 20,000 g for 20 min. The soluble fraction was transferred to a new tube and the glassy pellet of membranes was gently washed with lysis buffer, followed by addition of 400 μl of lysis buffer and resuspension of the membrane fraction by sonication in a water bath sonicator. The resuspended membranes were then subjected to another round of centrifugation, washing and resuspension, resulting in a washed membrane fraction, and 20 μg of protein of each fraction (cell lysate, soluble, and membranes) were subjected to SDS–PAGE on a 10% gel, followed by Coomassie staining or Western blotting with anti-His₉ antibody (Qiagen) at a dilution of 1:3000.

**In vitro activity assays of RsBtaA***

*Escherichia coli* TOP10 F’ harboring pBtaA was grown in 250 ml LB-ampicillin at 37 °C to an OD₆₀₀ of 0.7, and induced with 0.25 mM IPTG followed by an additional 4 h of growth at 28 °C. Cells were harvested by centrifugation and the cell pellet was suspended in 10 ml of cold buffer (50 mM Hepes, 1 mM DTT, 1 mM EDTA, pH 7.3). The resuspended cells were sonicated 3–4 times, 30 s each with a microprobe tip, and the lysate was centrifuged at 20000 g for 10 min to remove unbroken cells and cellular debris. Aliquots (1 ml) of the cell-free extract were then frozen in liquid N₂ and stored at −80 °C prior to use. Activity under these storage conditions did not decrease appreciably for at least 1 month.

Assays were conducted in 100 μl final volume by combining 48.75 μl of cell-free extract with 48.75 μl of 100 mM trion of each culture was then harvested by centrifugation and suspended in 10 ml fresh Sistrom’s medium containing 18.5 kBq 1-[¹⁴C]Met, followed by 3 h incubation at 28 °C, harvesting by centrifugation, and extraction as described for the *E. coli* cultures above. A portion of the organic phase from each extraction was applied to activated (120 °C, 2 h), ammonium sulfate impregnated TLC plates (Silica-60, Baker) and resolved in acetone/toluene/water (91:30:7, v/v) followed by autoradiography.

**Subcellular localization of BtaA protein***

A 2 ml overnight culture of cells containing pBtaA was used to inoculate 100 ml of LB-ampicillin, and then incubated at 37 °C to an OD₆₀₀ of 0.7–0.9. IPTG was added at 50 μM to induce low level expression of RsBtaA and to avoid the formation of inclusion bodies, and the culture was shifted to 28 °C and incubated further 4 h. Cells were harvested by centrifugation and suspended in 4 ml lysis buffer (20 mM KPi, pH 7.2, 500 mM NaCl, 15% (v/v) glycerol), frozen in liquid nitrogen and thawed slowly, and then lysed by sonication for 3 times, 30 s with a microprobe tip. The crude lysate was centrifuged at 20000 g for 10 min to remove unbroken cells and cellular debris, and the supernatant was subjected to centrifugation at 20,000 g for 20 min. The soluble fraction was transferred to a new tube and the glassy pellet of membranes was gently washed with lysis buffer, followed by addition of 400 μl of lysis buffer and resuspension of the membrane fraction by sonication in a water bath sonicator. The resuspended membranes were then subjected to another round of centrifugation, washing and resuspension, resulting in a washed membrane fraction, and 20 μg of protein of each fraction (cell lysate, soluble, and membranes) were subjected to SDS–PAGE on a 10% gel, followed by either Coomassie staining or Western blotting with anti-His₉ antibody (Qiagen) at a dilution of 1:3000.

**Table 1** Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td><strong>R. sphaeroides</strong></td>
<td>Wild-type</td>
<td>[36]</td>
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<tr>
<td>2.4.1</td>
<td>2.4.1 with Kan’ cassette disrupting btaB</td>
<td>[13]</td>
</tr>
<tr>
<td>btaB-dis</td>
<td>Cloning and expression strain, Φ 80 lacZ A M15, F’ (lacF’ Tn10[TetR])</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>TOP10F’</td>
<td>Cloning and expression strain,</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>PCR product cloning vector (AmpR)</td>
<td>Stratagene (La Jolla, CA, USA)</td>
</tr>
<tr>
<td>pCR2.1</td>
<td>General cloning vector</td>
<td>La Jolla, CA, USA</td>
</tr>
<tr>
<td>pBlueScript</td>
<td>pRKL323 R. sphaeroides btaAB operon in pBlueScript SK+</td>
<td>[13]</td>
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<tr>
<td>SK+</td>
<td>Low copy <em>E. coli</em> expression vector (CamR)</td>
<td>[15]</td>
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<tr>
<td>pACYC-31</td>
<td>High copy <em>E. coli</em> expression vector (AmpR)</td>
<td>Qiagen</td>
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<td>pQE-31</td>
<td>Split-KpnI fragment of btaA amplified from pRKL323 in pQE-31</td>
<td>This work</td>
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<tr>
<td>pBtaA</td>
<td>BamHI–HindIII fragment of btaB amplified from pRKL323 in pACYC-31</td>
<td>This work</td>
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PCRs and qPCRs were propagated in LB medium containing ampicillin at 100 μg/ml, and pACYC-31 derivatives in LB with chloramphenicol at 25 μg/ml. Strains containing compatible combinations of plasmids as indicated in individual experiments were constructed by co-transformation of heat-shock competent cells of TOP10F’ (Invitrogen) with 100 ng of both plasmids followed by selection on LB containing both ampicillin and chloramphenicol.
Hepes, Tris–Cl, or MES, 1 mM DTT, 1 mM EDTA, at varying initial pH to give a final pH in the range of 5.5–8.6 when mixed with the cell-free extract (initial, pH 7.3). Reactions were initiated by addition of 25,000 dpm 1-[14C]AdoMet (American Radiolabeled Chemicals, 2.5 μl, final concentration of AdoMet of 2.1 μM, specific activity 7.14 MBq/nmol), or 75,000 dpm [14C]DAG (dioleoyl-rac-glycerol, [oleoyl 1-14C]; American Radiolabeled Chemicals, final concentration 6.3 μM, specific activity 7.14 MBq/nmol). For the reaction initiated with labeled DAG, it was essential to dry [14C]DAG, which was delivered suspended in toluene/ethanol (1:1, v/v) at room temperature under a stream of nitrogen. A sonicating water bath was used to disperse the DAG at the desired concentration in a buffer of 50 mM Hepes, pH 7.8, 1 mM EDTA, 0.1% Triton X-100. Reactions were incubated for 30 min at 28 °C, and terminated by addition of 400 μl of chloroform/methanol (1:1, v/v) at room temperature under a stream of nitrogen. A sonicating water bath was used to disperse the DAG at the desired concentration in a buffer of 50 mM Hepes, pH 7.8, 1 mM EDTA, 0.1% Triton X-100. Reactions were incubated for 30 min at 28 °C, and terminated by addition of 400 μl of chloroform/methanol (1:1, v/v) at room temperature under a stream of nitrogen. A sonicating water bath was used to disperse the DAG at the desired concentration in a buffer of 50 mM Hepes, pH 7.8, 1 mM EDTA, 0.1% Triton X-100. Reactions were incubated for 30 min at 28 °C, and terminated by addition of 400 μl of chloroform/methanol (1:1, v/v) at room temperature under a stream of nitrogen. A sonicating water bath was used to disperse the DAG at the desired concentration in a buffer of 50 mM Hepes, pH 7.8, 1 mM EDTA, 0.1% Triton X-100. 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Sistrom’s medium to induce synthesis of DGHS in the *btaB-dis* strain, or DGTS in the wild-type. We expected that *E. coli* expressing *Rs* *BtaA* could use endogenous diacylglycerol (DAG) and AdoMet to accumulate DGHS, the non-methylated precursor of DGTS (Fig. 1). In Fig. 2A, we show that this was indeed the case, as a primary-amine containing lipid which co-migrated with authentic DGHS was present upon expression of *btaA* in *E. coli*. This result firmly established the role of *Rs* *BtaA* as the enzyme catalyzing the first step of DGTS biosynthesis in the pathway outlined in Fig. 1, and excluded the possibility that *Rs* *BtaA* acts as a component of a heteromeric complex with a protein specific to *R. sphaeroides*, or that it requires a substrate or co-factor present in *R. sphaeroides* only.

To show the function of the putative trifunctional methyltransferase *Rs* *BtaB*, a compatible expression plasmid containing the *Rs* *btaB* gene was introduced into *E. coli* along with pBtaA. Our expectation was that a portion of the DGHS produced by *Rs* *BtaA* would be converted into N-methylated products as shown in Fig. 1. Fig. 2B shows the results of a metabolic labeling strategy aimed at testing the function of *Rs* *BtaB*, *R. sphaeroides* or *E. coli* strains were incubated with 1-[^14]C]methionine, which is taken up and converted into AdoMet by the action of the AdoMet synthetase MetK [19], and then used by *Rs* *BtaA* for DGHS synthesis. The fate of the radiolabel was determined by analysis of the resulting lipids by TLC and autoradiography. The pBtaA/pACYC construct lead to the accumulation of label in DGHS, and in the pBtaA/pBtaB strain a portion of DGHS was converted to DGTS by the action of *Rs* *BtaB*, as judged by co-migration of the new species with authentic DGTS. A representative result using the His6-tag, but our attempts were

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**Fig. 3.** The *Rs* *BtaA* protein is associated with cellular membranes. (A) Hydrophathy analysis of *Rs* *BtaA*, B, and C, *E. coli* cells harboring pBtaA or empty pQE-31 vector were induced, harvested, and separated into a soluble and membrane fraction as described in the text. Twenty micrograms of protein of each fraction were electrophoresed on a 10% SDS-PAGE gel and (B) stained with Coomassie blue or (C) transferred to a PVDF membrane and probed with anti-His tag antibody. The position of His$_6$-BtaA protein (~45 kDa) is indicated.

(Rs) *BtaA is associated with membranes*

Analysis of the localization of *Rs* *BtaA* within the *E. coli* cell is given in Fig. 3. Predictions based on hydrophathy analysis (Fig. 3A), [20] and with the TMHMM algorithm [21] (not shown) suggested that *Rs* *BtaA* is devoid of membrane spanning helices and is very hydrophilic throughout. Based upon this analysis, *Rs* *BtaA* was predicted to be a soluble protein. Fig. 3B shows the results of the fractionation of a *Rs* *btaA*-expressing *E. coli* culture by differential centrifugation with the parental pQE-31 vector serving as control. Comparison of the cell lysate (Fig. 3B, left two lanes) and soluble (Fig. 3B, middle two lanes) fractions showed no discernable difference between the two protein profiles, but the membrane fraction (right two lanes) was enriched in the ~45kDa putative *Rs* *BtaA* protein in the pBtaA membrane fraction (Fig. 3B, arrowhead). The His$_6$-tagged recombinant *Rs* *BtaA* protein was also identified by immunoblotting with anti-His$_6$ antibody as shown in Fig. 3C. Empty vector controls were not present on the immunoblot shown, but were routinely devoid of cross-reacting bands. Apparently, *Rs* *BtaA* was present in the cell lysate and membrane fractions, but was absent from the soluble fraction, suggesting that it is a membrane-associated protein. The presence of multiple basic residues at the termini of some of the BtaA homologs may indicate that the protein is bound to the membrane through electrostatic interactions, similar to some peripheral membrane proteins, e.g., [22].

**Rs* *BtaA has AdoMet/diacylglycerol 3-amino-3-carboxypropyl transferase activity**

In vitro assays of *Rs* *BtaA* enzyme activity were conducted using 1-[^14]C]AdoMet or [^14]C]DAG to follow product formation and the results are presented in Fig. 4. Initially, we attempted to solubilize and purify *Rs* *BtaA* utilizing the engineered His$_6$-tag, but our attempts were
unsuccessful. While we were able to purify small amounts of apparently soluble RsBtaA protein using various detergents in the lysis buffer, we were unable to demonstrate activity in a reconstituted liposome system. To circumvent this problem, we developed a system to minimize the steps between expression and enzyme assay to demonstrate the proposed reaction catalyzed by RsBtaA. In general, E. coli cells contain small amounts of DAG derived from the synthesis of membrane-derived oligosaccharides [23] and the reaction will proceed without addition of DAG. However, as we wanted to test the incorporation of label from 1-[14C]AdoMet as well as [14C]DAG, two sets of controlled reactions were set up keeping all conditions the same except for the compound carrying the label. Reaction products were separated by TLC and detected using a phosphor-imager. The product bands containing DGHS (missing in the vector control) were shown inside the box. DAG, diacylglycerol; DGHS, diacetylglycerylhomoserine; Ori, origin.

Fig. 4. In vitro assay of BtaA activity. E. coli cells expressing RsBtaA were broken by sonication and assayed for the ability to incorporate label from 1-[14C]AdoMet or [14C]DAG into DGHS. Cells either contained the pBtaA plasmid or an empty pQE31 vector control (Vec). Time courses are shown with incubation times (min) indicated. Reaction products were separated by TLC and detected using a phosphor-imager. The product bands containing DGHS (missing in the vector controls) are shown inside the box. DAG, diacylglycerol; DGHS, diacetylglycerylhomoserine; Ori, origin.

Table 2

<table>
<thead>
<tr>
<th>[14C]-AdoMet</th>
<th>[14C]-DAG</th>
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<tbody>
<tr>
<td>pBtaA</td>
<td>Vec</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
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</table>

- DAG
- DGHS
- Ori

The vector control consistent with it being DGHS. When labeled DAG was used, label was observed in a number of polar lipids as DAG is a general precursor for polar lipid biosynthesis. These labeled compounds were also present in the vector control. One compound co-chromatographing with DGHS present in the AdoMet reaction (Fig. 4, box) was also present, but unlike other lipids, it was absent from the vector control thereby suggesting that it was DGHS. Thus, labeled DAG appeared to serve as a precursor for DGHS biosynthesis under the employed conditions. To determine the relative rates of incorporation, the phosphor imager outputs were quantified. The rates were linear for both labeled compounds (r² = 0.9964 for the AdoMet reaction and r² = 0.9743 for the DAG reaction) over the incubation time suggesting that the enzyme was present at non-saturating amounts in the reaction mixture. The rates were very similar (29.6 relative units/min for the AdoMet reaction and 27.7 relative units/min for the DAG reaction). Given that the specific activity for the labeled compounds and the total substrate concentrations in both reactions were identical, one can cautiously conclude that both compounds were incorporated into DGHS with similar efficiency as expected for the direct substrates of the reaction.

To independently corroborate that DAG is indeed the lipid substrate of BtaA, the cell lysate was incubated with active or heat inactivated phospholipase C (PLC), thus generating DAG in situ. In this case, DAG was not added to the reaction mixture and labeled AdoMet was used to follow the reaction. Under these conditions, RsBtaA activity was stimulated at least twofold in the assay mixtures (data not shown) that were pretreated with 10 U of active PLC, consistent with a role of DAG as the lipid substrate.

A number of divalent metals including Mg²⁺, Ca²⁺, and Mn²⁺ were tested at 1 mM for their ability to stimulate the reaction, but no stimulatory effect was observed, with the possible exception of slight increases in activity by calcium ions (data not shown). This stimulation was variable between different preparations of enzyme, indicating that if Ca²⁺ does have a stimulatory effect, it might be indirect, e.g., Ca²⁺ could act as a counter ion to membrane phospholipids and thus stabilize the membrane upon which RsBtaA is acting. The metal chelators EDTA and EGTA were tested and were found to have no effect on enzyme activity, corroborating the idea that RsBtaA is probably not a metalloenzyme. RsBtaA enzyme activity was found to have a slightly basic pH optimum of ~7.6 (Fig. 5A), and the apparent K_M toward AdoMet was 27 μM (Fig. 5B).

BtaA is related to methyltransferases

As shown in the multiple sequence alignment of Fig. 6A, BtaA from R. sphaeroides has orthologs in a number of other bacteria, predominantly its relatives in
the Gram-negative family of x-proteobacteria. The BtaA orthologs from *S. meliloti* and *Agrobacterium tumefaciens* were cloned into pQE-31 essentially as described above for *btaA* from *R. sphaeroides*, and recombinant *E. coli* carrying these constructs also accumulated DGHS (data not shown), indicating that the bacterial BtaA proteins are truly homologous in both sequence and function. All of these BtaA orthologs are encoded in a putative operon of the same structure as that of *R. sphaeroides*, which is predicted to be transcribed as a bicistronic *btaAB* message [13], except for the BtaA-like protein of *Xylella fastidiosa*, which is not accompanied by a *btaB* gene in the predicted operon. Given the fact that BtaB is similar to other small-molecule methyltransferases, it was impossible to unequivocally identify the BtaB ortholog elsewhere in this genome. Using PSI-BLAST [24] to search for other protein families that might be related to BtaA, we discovered a number of related, but as yet uncharacterized bacterial methyltransferase-like proteins. A sequence alignment of the pertinent regions, as well as an analysis of putative motifs involved in AdoMet binding [25] is shown in Fig. 6B, leading to a prediction of residues involved in AdoMet binding, as presented schematically in Fig. 7B.

Discussion

This work was undertaken to determine the biochemical function of two proteins, BtaA and BtaB, which had recently been shown by genetic means to be necessary for phosphate starvation-induced DGTS biosynthesis in *R. sphaeroides* [13]. A key result presented here is the demonstration that, not only are BtaA and BtaB necessary for DGTS biosynthesis in *R. sphaeroides*, but that they are also sufficient to provide all the activities in the biosynthetic pathway, as judged by their ability to convey DGTS production in *E. coli*, which normally lacks this lipid. Genetic analysis following the isolation of these genes left this point unresolved, in that disruption of the *rsbtaB* gene with a kanamycin resistance cassette resulted in the accumulation of both DGHS and a monomethylated form during phosphate starvation, leaving the possibility open that the *RsBtaB* protein may only act as the methyltransferase for the second and/or third N-methylation(s) of the precursor [13]. While low expression of *RsBtaB* sufficient for DGTS biosynthesis was achieved in *E. coli*, it was not possible to purify the active *RsBtaB* protein in amounts permitting further characterization.

However, the *RsBtaA* protein in a crude membrane preparation was shown to transfer the 3-amino-3-carboxypropyl moiety from AdoMet to DAG by demonstrating that under identical conditions incorporation of label into DGHS from either labeled AdoMet or DAG proceeded at the same rate (Fig. 4). This result is in agreement with the role of *RsBtaA* that had been tentatively assigned based on mutagenesis and ectopic expression, and rules out other contingencies for the activity of *RsBtaA*. Attempts to solubilize, purify, and reconstitute BtaA into liposomes of defined composition were unsuccessful. In fact, the BtaA activity was sensitive to low concentrations of detergent when, e.g., F 0.5% (w/v) octyl-glucoside or Tween 20 was added to an assay as described above. For this reason, we took the approach of keeping the recombinant enzyme in as native a state as possible and minimizing the steps between expression and enzyme assay.

Previous work in a cell-free system on the cognate BtaA-type transferase activity in *C. reinhardtii* using labeled AdoMet to follow the reaction had given conflicting results as to the identity of the hydrophobic substrate, indicating that DAG might not be the direct substrate because the addition of unlabeled DAG strongly inhibited the reaction [26]. However, the point was raised that excess DAG might simply disrupt the membrane environment in which the enzyme(s) are working, and the decrease in activity might not be a result of enzyme inhibition, per se. The results we present for recombinant *RsBtaA* seemingly provide a solution to this question, in that incubation of this enzyme/membrane system with PLC stimulated the rate of DGHS...
Fig. 6. Multiple sequence alignment of bacterial BtaA orthologs and methyltransferases. (A) BLAST searching using R. sphaeroides BtaA (Rsp, AAK53560) as query was used to identify orthologous proteins in finished genomes of A. tumefaciens (Atu, NP_355081), S. mellioti (Sme, NP_386300), Mesorhizobium loti (Mlo, NP_103130), Rhodopseudomonas palustris (Rpa, NP_946080), Pirellula sp. (Psp, NP_863860), X. fastidiosa (Xfa, NP_299322), and Bdellovibrio bacteriovorus (Bba, NP_969921). Arrow heads indicate highly conserved amino acids G75 and D94. (B) PSI-BLAST revealed that a domain in the N-terminal half of BtaA is similar to the AdoMet binding motifs of some bacterial methyltransferases. Regions corresponding to methyltransferase motifs I and II are indicated with (**), and GenBank protein accession numbers are used as identifiers for the methyltransferases in the pileup. Solid lines over the aligned proteins at the N- and C-termini are predicted to be polybasic stretches or basic-amphiphatic helices mediating membrane binding, as has been described for other proteins.
The kinetic properties of \( \text{RsBtaA} \) toward AdoMet and its analogs are similar to those of methyltransferases. The apparent \( K_M \) for AdoMet of 27 \( \mu \)M that was measured for \( \text{RsBtaA} \) is in line with values obtained for methyltransferases, which range from \( \sim 5 \) to 50 \( \mu \)M [27–29]. \( \text{RsBtaA} \) was weakly inhibited by S-adenosylhomocysteine, which lacks the activated methyl unit of AdoMet and competitively inhibits methyltransferases [30], and also by its presumed product, 5’-methylthioadenosine, but not by methionine itself (data not shown). The pH profile showed an optimum at \( \sim \text{pH 7.6} \), which is similar to that measured for the activity from \( C. \text{reinhardtii} \) [26,31].

Having established that \( \text{RsBtaA} \) is active in the proposed reaction, and given that the substrate DAG is a minor component of bacterial cell membranes [23] we wanted to determine if \( \text{RsBtaA} \) associates with membranes. Hydropathy analysis using a standard Kyte–Doolittle algorithm [20] (Fig. 3A) or a more sensitive hidden-Markov model based approach (TMHMM) [21] to predict transmembrane helices revealed that \( \text{RsBtaA} \) is actually quite hydrophilic and predicted to be a soluble protein. However, fractionation by differential centrifugation (Figs. 3B and C) revealed that it is probably a peripheral membrane protein, as it was not present in a soluble form in preparations that are active in the \( \text{RsBtaA} \) assay, but was instead present solely in membrane-containing fractions. Analysis of the multiple sequence alignment in Fig. 6A showed that a segment of 12–15 basic and hydrophobic residues is present in the N-terminus of some of the homologs, as well as a polybasic stretch at the C-terminus. This finding led to the hypothesis that these regions might target and anchor \( \text{RsBtaA} \) to the inner membrane by forming a basic-amphipathic \( \alpha \)-helix and providing favorable electrostatic interactions with the anionic membrane surface, as is the case for some other peripheral membrane proteins [22,32].

The \( \text{RsBtaA} \) enzyme is a member of a small group of enzymes using the ubiquitous alkylating agent AdoMet as a donor of a 3-amino-3-carboxypropyl moiety rather than the methyl group. Other characterized enzymes that catalyze a similar reaction include one involved in antibiotic biosynthesis in \( N. \text{uniformis} \) [33], and spermidine synthase, which transfers the aminopropyl moiety of decarboxylated-AdoMet to putrescine in the polyamine biosynthesis pathway [34]. Sequence and structural similarity of these enzymes to AdoMet-dependent methyltransferases has been demonstrated [33,34] and it is clear from these results that these proteins share a methyltransferase fold [25,30] with a conserved AdoMet binding pocket. The identity of BtaA as a methyltransferase fold protein was not readily apparent on the basis of sequence analysis, but a detailed comparison of the bacterial BtaA orthologs allowed the identification of putative AdoMet binding motifs. The multiple sequence alignments in Figs. 6A and B show that residues corresponding to G75 and D94 in the \( R. \text{sphaerooides} \) protein are completely conserved among the BtaA homologs, and correspond to the conserved residues in AdoMet binding motifs I and II, analogous to the “G-loop” and “D-loop” in other nucleotide binding motifs [25,35], providing evidence that BtaA also binds AdoMet in a methyltransferase fold. A schematic representation of the active site of BtaA is presented in Fig. 7B, and a model for the predicted topology and binding sites of DAG and AdoMet are represented in Fig. 7A. The involvement of a hypothetical general base in activating the nucleophilic hydroxyl of DAG is also presented in
Fig. 7A, however, the presence and/or identity of this residue was not predictable from the sequence analysis given the very limited similarity of BtaA to methyltransferases outside of the putative AdoMet binding domain. Taken together, the results provide first direct insight into the enzymatic mechanisms of betaine lipid biosynthesis in bacteria and identify the key step in the pathway, DGHS formation, as being catalyzed by a highly divergent methyltransferase-like enzyme.

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References