Rhodobacter capsulatus OlsA Is a Bifunctional Enzyme Active in both Ornithine Lipid and Phosphatidic Acid Biosynthesis

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The Rhodobacter capsulatus genome contains three genes (olsA [plsC316], plsC316, and plsC3498) that are annotated as lysophosphatidic acid (1-acyl-sn-glycerol-3-phosphate) acyltransferase (AGPAT). Of these genes, olsA was previously shown to be an O-acyltransferase in the second step of ornithine lipid biosynthesis, which is important for optimal steady-state levels of c-type cytochromes (S. Aygun-Sunar, S. Mandaci, H.-G. Koch, I. V. J. Murray, H. Goldfine, and F. Daldal. Mol. Microbiol. 61:418–435, 2006). The roles of the remaining plsC316 and plsC3498 genes remained unknown. In this work, these genes were cloned, and chromosomal insertion-deletion mutations inactivating them were obtained to define their function. Characterization of these mutants indicated that, unlike the Escherichia coli plsC, neither plsC316 nor plsC3498 was essential in R. capsulatus. In contrast, no plsC316 olsA double mutant could be isolated, indicating that an intact copy of either olsA or plsC316 was required for R. capsulatus growth under the conditions tested. Compared to OlsA null mutants, PlsC316 null mutants contained ornithine lipid and had no c-type cytochrome-related phenotype. However, they exhibited slight growth impairment and highly altered total fatty acid and phospholipid profiles. Heterologous expression in an E. coli plsC(Ts) mutant of either R. capsulatus plsC316 or olsA gene products supported growth at a nonpermissive temperature, exhibited AGPAT activity in vitro, and restored phosphatidic acid biosynthesis. The more vigorous AGPAT activity displayed by PlsC316 suggested that plsC316 encodes the main AGPAT required for glycerophospholipid synthesis in R. capsulatus, while olsA acts as an alternative AGPAT that is specific for ornithine lipid synthesis. This study therefore revealed for the first time that some OlsA enzymes, like the enzyme of R. capsulatus, are bifunctional and involved in both membrane ornithine lipid and glycerophospholipid biosynthesis.
FIG. 1. PA and OL biosynthesis pathway in bacteria. (A) The first step for PA biosynthesis from G3P can be carried out by two different routes. In some bacteria, like E. coli, GAPAT (PlsB) acylates the sn-1 position of G3P using either acyl-ACP or acyl-CoA to form LPA (step 1). In other bacteria, a recently identified route uses the soluble PlsX to convert acyl-ACP to acyl-phosphate (acyl-P), followed by the membrane-associated PlsY transferring the acyl chain to G3P (step 2). In all bacteria, the second step for PA biosynthesis is catalyzed by the membrane-associated AGPAT (PlsC) enzyme, which transfers an acyl chain from either acyl-ACP or acyl-CoA to LPA to yield PA. In R. capsulatus OlsA is alternative AGPAT enzyme for production of PA. (B) During OL biosynthesis, the first enzyme OlsB catalyzes the formation of an amide linkage (N-acyltransferase) between the α-amino group of ornithine and the carboxyl group of a 3-hydroxy fatty acid, forming LOL. The second enzyme, OlsA catalysts the formation of an ester linkage (O-acyltransferase) between the 3-hydroxy group of the fatty acid group and the carboxyl of a second fatty acid, converting LOL to OL.

Bacterial strains and growth conditions. The bacterial strains and plasmids used are listed in Table 1. E. coli strains were grown aerobically in LB medium (35), and R. capsulatus strains were grown at 35°C in either minimal medium A (MedA) (43) or enriched (MPYE) medium supplemented with appropriate antibiotics, as described previously (36). The ability of various R. capsulatus genes to complement the growth defect of a temperature-sensitive E. coli PlsC(Ts) mutant (11) was tested by monitoring growth at 42°C on LB plates supplemented with ampicillin (100 mg/ml) and 0 to 2% L-arabinose, as appropriate. The ability of R. capsulatus genes to complement an E. coli PlsB− mutant was tested by monitoring growth at 37°C the G3P autophosphorylation of appropriate derivatives of strain SJ22 (pMB26 pMB30) (39) on minimal medium E (35) plates supplemented with 2% L-arabinose (2%), ampicillin (100 mg/ml), and 0.4% G3P, as needed.

Molecular genetic techniques. Standard molecular biological techniques were performed according to Sambrook et al. (40) and Daldal et al. (15). Homology searches and amino acid sequence alignments were done using MacVector (Accelyers, San Diego, CA) and appropriate software programs as described earlier (36).

The plsC316 gene (annotated RRC0316 on the R. capsulatus genome) was cloned by PCR amplification using chromosomal DNA and the primers 5′-AA GCTCTAGATTCGCGCGCAGCGCTAGATGAAA-3′ and 5′-CAGCCTGACCCGGCGTGCAGACGAAAAGTCCT-3′ containing the XbaI and KpnI sites (in boldface), respectively. The latter plasmids were conjugated into the GTA overexpression strain MT1131, the single mutants SA11 [::plsC316] and SA13 [::plsC3498] in the wild-type strain MT1131, the double mutants SA12 [::plsC316::plsB26::plsX50] (Table 1) were obtained. Similarly, the double mutants SA12 [::plsC316::plsB26::plsX50] in strain SJ22 (pMB26 pMB30) on minimal medium E (35) plates supplemented with 2% L-arabinose (2%), ampicillin (100 mg/ml), and 0.4% G3P, as needed.

Expression of plsC316 and plsC3498 in E. coli. The plsC gene was PCR amplified using the primers sPCR1 (28) (Table 1) as a template and the primer pairs olsA::NcoI (5′-GGAGCGCCCCCATGCTGCAAGCTGATCG-3′) and olsA::EcoRI (5′-CTGGCGGCAATTCGCGGACGCGTCC-3′) containing the Neol and EcoRI restriction enzymes sites (in boldface), respectively. The plsC316 gene was amplified using the plasmid pSEM21 (Table 1) as a template and the primers

human, and mouse as well (1, 8, 20, 26, 31, 32, 44, 49, 50). In bacteria, the AGPATs also play a role in regulating lipid acyl composition through their substrate specificities (14, 42). Inactivation of one of the multiple plsC genes often alters fatty acid profiles of phospholipids and their membrane properties (14, 42).

Prior to this study, despite the broad importance of AGPATs, only limited knowledge was available on these enzymes, especially those from photosynthetic purple bacteria, including Rhodobacter species (C. Benning, personal communication). Earlier, we had isolated Rhodobacter capsulatus mutants that are defective in maintaining optimal steady-state levels of c-type cytochromes (cyt) (28). Studies of these mutants led us to the identification of olsA and olsB genes responsible for the biosynthesis of membrane ornithine lipid (OL) in R. capsulatus (3) (Fig. 1B). Initially, olsA was misannotated as plsC138 encoding an AGPAT homologue based on its high degree of similarity to acyl-acyltransferases (http://www.ergo-light.com). Mutants lacking an active olsA (or olsB) were unable to produce OL, but they contained a full complement of membrane glycerophospholipids, including PE, PG, and phosphatidylcholine (PC) (3). Thus, PA production must be carried out by an unknown enzyme distinct from OlsA. A whole-genome sequence revealed that the R. capsulatus chromosome contained two additional open reading frames (ORFs), annotated plsC316 and plsC3498, as candidates for an AGPAT enzyme involved in PA biosynthesis. In this work, we demonstrate that the plsC316 product is specific for only PA and not OL biosynthesis and that plsC3498 is involved in neither of these two pathways. We also show that the R. capsulatus olsA product is a bifunctional O-acyltransferase involved in both OL and PA biosynthesis. Furthermore, our findings indicate that while R. capsulatus plsC316 is likely to encode the primary AGPAT enzyme involved in PA biosynthesis, OL synthesis-specific olsA can also act as an alternate AGPAT to ensure glycerophospholipid production.
The PCR products obtained were digested with appropriate restriction enzymes, cloned into the corresponding sites of the expression vector pBAD/Myc-His A, yielding pSEM17 (Myc), pSEM25 cloned into XbaI-KpnI sites of pBAD/Myc-His A, or cloned into XbaI-KpnI sites of pBAD/Myc-His A on pSEM21 replaced the source of pSEM21.

**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Relevant characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong> E. coli</td>
<td>HB101: F' Δ(gpt-proA)62 leuB6 supE44 ara-14 galK2 lacY1 Δ(mcrC- mrr) pSL20 xyl-5 mtl-1 recA13</td>
<td>Strr; r_{H}^- m_{H}^-</td>
<td>40</td>
</tr>
<tr>
<td>TOP10: F' mcrA Δ(mrr-hsdRMS-mcrBC) Δ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ araE167)7697 galU galK rpsL endA1 supG</td>
<td>Strr; cloning host</td>
<td>Invitrogen</td>
<td></td>
</tr>
<tr>
<td>XL-1 Blue: F' ΔproAB lacIqZΔM15 Tn10</td>
<td>Tet; cloning host</td>
<td>Stratagene</td>
<td></td>
</tr>
<tr>
<td>SM2-1: pLSClmetC162::Tn10 thr-1 ara-14 Δ(gal-attHisG4) rpsL136 xyl-5 mtl-1 lacY1 txy-78 ada-50 rfdD1 thi-1</td>
<td>Tetr; Thrr</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>SJ22: pLSB26 plx50 panD2 zac-220Δ::Tn10 gpdD3 gplK rplA1 rpoT1 pit-10 phaA8 ompF627 fluA22 fosL701</td>
<td>Auxotrophic for G3P on medium E</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>

**R. capsulatus**

| MT131: crtD121 Rif^- | Wild type | 28 |
| Y262 | GTA overproducer | 51 |
| SA4 | Δ(olsA::spe) | 3 |
| SA11: plsC3498::gm | Gmr^- | This study |
| SA12 | Δ(olsA::spe) | Gmr^- Spe^- | This study |
| SA13 | Δ(olsC316::kan) | Kan^- | This study |
| SA14 | Δ(olsC316::kan) | Gmr^- Kan^- | This study |
| SA15 | Δ(olsC316::kan) harboring intact olsA on the plasmid | Spe^- Kan^- Tetr^- | This study |
| SA16 | Δ(olsC316::kan) harboring intact plsC316 on the plasmid | Spe^- Kan^- Tetr^- | This study |

**Plasmids**

| pRK2013: tra^- (RK2) | Kan^-; conjugative helper | 19 |
| pRK415 | Tet^-; broad-host-range vector | 18 |
| pBSII | pBluescript II (K(S+)) | Amp^- | Stratagene |
| pMA117: ΩKan | Kan^- | 15 |
| pCHB::Gm^- | Gm^- Tetr^- | K. Zhang and F. Daldal |
| pBAD/Myc-His A | Amp^-; arabinose-inducible vector | Invitrogen |
| pMRC | 6-kb chromosomal EcoRI fragment containing olsA in pLAFR1 | Tet^- | 28 |
| pSEM11 | Δ(olsA::spe) | Tet^- Spe^- | 19 |
| pDM1 | 657-bp PCR product containing plsC3498 cloned into Ncol- EcoRI sites of pBAD/Myc-His A | Amp^- | This study |
| pDM3 | 768-bp PCR product containing plsC3498 cloned into Xbal-KpnI sites of pBSII | Amp^- | This study |
| pDM4 | Xbal-HindII-Gm of pCHB::Gm inserted into unique SmaI site of plsC3498 on pDM3 | Amp^- Gm^- | This study |
| pSEM17 | 828-bp PCR product containing olsA cloned into Ncol-EcoRI sites of pBAD/Myc-His A | Amp^- | This study |
| pSEM18 | NsiI-cut pSEM17 ligated into PstI-cut pRK415 | Tet^- Amp^- | This study |
| pSEM21 | 1.9-kb PCR product containing plsC316 cloned into Xbal-KpnI sites of pBSII | Amp^- | This study |
| pSEM24 | 1.9-kb Xbal-KpnI fragment of pSEM21 cloned into Xbal-KpnI sites of pRK415 | Tet^- | This study |
| pSEM25 | 819-bp PCR product containing plsC316 cloned into Ncol-SfuI sites of pBAD/Myc-His A | Amp^- | This study |
| pSEM26 | NsiI-cut pSEM25 ligated into PstI-cut pRK415 | Tet^- Amp^- | This study |
| pSEM27 | NsiI-cut pDM1 ligated into PstI-cut pRK415 | Tet^- Amp^- Kan^- | This study |
| pSEM30 | 578-bp ThrI111-RrsII fragment of plsC316 on pSEM21 replaced with Sall-Ωkan of pMA117 | Tet^- Kan^- | This study |
| pSEM31 | 2.2-kb BglII fragment of pSEM31 ligated to HindIII-KpnI sites of pRK415 | Tet^- Kan^- | This study |
| pSEM35 | 1.570-bp PshAI-KpnI fragment of pDM4 cloned into KpnI sites of pRK415 | Gmr^- | This study |

*Abbreviations of antibiotic resistances are as follows: Amp, ampicillin; Gm, gentamicin; Kan, kanamycin; Rif, rifampin; Spe, spectinomycin; Str, streptomycin; Tet, tetracycline.*
(pksC316), and pDM1 (pksC3498), respectively (Table 1). The resulting plasmids were sequenced to confirm that olsA, pksC316, and pksC3498 were in frame with the vector’s translation start site and were epitope tagged at their carboxyl termini. Automated DNA sequencing with a BigDye terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA) was used with the primers pBAD-Seq-F (5’-ATGCTACTACGTTTACCTT-3’) and pBAD-Seq-R (5’-GATTTCACGTGATACGG-3’). Derivatives of the transferable plasmid pRK415 carrying olsA, pksC316, or pksC3498 were constructed by cloning the 4.9-kb NsiI fragment of pSME17, the 4.9-kb NsiI fragment of pSME25, and 4.8-kb NsiI fragment of pDM1 into the PstI site of pRK415 to generate pSEM18, pSEM26, and pSEM27, respectively. Conjugal transfer of all plasmids from E. coli to R. capsulatus was carried out as described earlier (18).

Expression of R. capsulatus pscC homologues in either E. coli or R. capsulatus. To monitor the expression of olsA, pksC316, or pksC3498, the plasmids pSME17 (olsA), pSME25 (pksC316), or pDM1 (pksC3498), respectively, were transformed into the E. coli strain SM2-1 [pCts(Ts)] (11). Appropriate derivatives of SM2-1 were grown to an optical density at 600 nm (OD600) of ~0.5, and cultures were induced for 4 h with increasing amounts (0 to 2%) of 3-aminobenzylphosphonate. In each case 1 ml of cell culture was collected by centrifugation, and the whole-cell membranes were prepared (34). The reaction mixture with a 5-min incubation at 37°C was also run to monitor LPA and PA production using thin layer chromatography (TLC). At the end of the incubation period, 2 ml of chloroform:methanol:water (14:6:1, vol/vol/vol) were applied to a preheated silica gel G60 TLC plate. Plates were developed with chloroform:methanol-water (14:6:1, vol/vol) and chloroform-methanol-glacial acetic acid (13:5:2, vol/vol/vol) for the first and second dimensions, respectively (16). Radioabeled lipids were visualized, identified, and quantified as described above. Fatty acid compositions of appropriate R. capsulatus strains were determined using approximately 30 mg of wet cell pellets grown in MPYE medium, and fatty acid methyl ester analysis was carried out by MIDI Inc. (Newark, DE).

The following compounds were used in the bacterial experiments: 35°C) was also run to monitor LPA and PA production using thin layer chromatography (TLC). At the end of the incubation period, 2 ml of chloroform:methanol:water (14:6:1, vol/vol/vol) were applied to a preheated silica gel G60 TLC plate. Plates were developed with chloroform:methanol-water (14:6:1, vol/vol) and chloroform-methanol-glacial acetic acid (13:5:2, vol/vol/vol) for the first and second dimensions, respectively (16). Radioabeled lipids were visualized, identified, and quantified as described above. Fatty acid compositions of appropriate R. capsulatus strains were determined using approximately 30 mg of wet cell pellets grown in MPYE medium, and fatty acid methyl ester analysis was carried out by MIDI Inc. (Newark, DE).

Chemicals, reagents, and enzymes. Restriction enzymes, oligonucleotide primers, [1-14C]acetate (150 mCi mmol⁻¹ specific activity) and [1-14C]acetate (60 mCi mmol⁻¹ specific activity) were purchased from New England Biolabs, the Cell Center facility of the University of Pennsylvania, American Radiolabeled Chemicals Inc., and NEN Life Science Products, respectively. ACP was obtained from either Sigma Chemical Co. or Invitrogen Inc. cis-Vaccenic Acid, G3P, and LPA were from Sigma Chemical Co.; PA was from Avanti Polar Lipids; and DEAE cellulose (DE52) was from Whatman. All other chemicals were from commercial sources and of highest available purity.

RESULTS

Identification of two additional pscC homologues in R. capsulatus genome. Our previous work established that OlsA null mutants lack only ORL; are not lethal, unlike an E. coli PlsC mutant; and still produce adequate levels of PE, PC, and PG (3). These findings indicated that in the absence of olsA, R. capsulatus must have other means of producing PA, which is an essential intermediate for membrane glycerophospholipid biosynthesis. A survey of the R. capsulatus genome (http://www.ergo-light.com) revealed two additional AGPAT candidates in addition to RCR00138, which was initially annotated as pscC316 but subsequently renamed olsA, acting as O-acetyltransferase engaged in OL synthesis (3). The ORFs RCR00316 (pksC316) and RCR03498 (pksC3498) were annotated as AGPAT homologues, and, like OlsA, they exhibited high degrees of similarities to the E. coli PlsC. They contained a conserved acetyltransferase (pfla01553/COG0204) motif and a highly conserved (HX_D) sequence thought to be common to AGPAT and AGPAT enzymes (24) (Fig. 2).

On the R. capsulatus chromosome, the two pscC homologues are located at different regions distant from each other and from olsA. pksC316 is 819 bp in length, encodes 273 amino acids, and is surrounded by the ORFs RRC00314, RRC00315, RRC00701, and RRC00317, corresponding to cdsA, accA, ftsX, and ftsE, respectively; pksC3498 is 657 bp in length, encodes 219 amino acids, and is surrounded by the ORFs RRC03495, RRC03496, RRC03497, and RRC03498 corresponding to acoB, acoC, cdaA, and cysE, respectively (see Fig. S1 in the supplemental material and the legend for a functional description of these genes). Multiple alignments of these ORFs illustrated their similarities to the E. coli PlsC and to each other (Fig. 2). For example, R. capsulatus OlsA, PksC316, and PksC3498 show 18%, 19%, and 19% identity and 24%, 32%, and 26% similarity to the E. coli PlsC, respectively. Note

BIFUNCTIONAL ACTIVITY OF R. CAPSULATUS OlsA

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that the highest degree of similarity is seen between the
R. capsulatus PlsC316 and E. coli PlsC. Moreover, plsC316 is also
flanked by cell division-related genes ftsX and ftsE (http://www
.ergo-light.com), like the E. coli plsC located between sufI
involved in cell division and parC encoding a topoisomerase
involved in chromosome partitioning (12). No similar synteny
between E. coli and R. capsulatus was observed for
olsA or plsC3498, which are located immediately downstream of
olsB, encoding an N-acyltransferase involved in OL biosynthesis (3),
or cdsA, encoding phosphatidate cytidylytransferase (RRC03497)
converting PA to CDP-diacylglycerol, respectively (see Fig. S1 in
the supplemental material).

Insertional inactivation of R. capsulatus plsC homologues
and characterization of ensuing mutants. The R. capsulatus
AGPAT homologues plsC316 and plsC3498 were cloned, and
their mutant alleles were constructed using interposon mu-
tagenesis, as described in Materials and Methods, in order to
define which one of them is responsible for PA biosynthesis in
R. capsulatus. The single mutants lacking an active PlsC316
(SA13 [plsC316::kan]) or PlsC3498 (SA11 [plsC3498::gm])
were obtained readily and compared with a mutant lacking an
active OlsA (SA4 [olsA::spe]). Unlike the E. coli PlsC
mutants that are lethal, neither plsC316 nor plsC3498 was
essential for growth of R. capsulatus under the photosynthetic
or respiratory conditions on MPYE or MedA growth medium.
However, it was noted that the PlsC316 mutant formed
slightly smaller colonies than the OlsA− or the PlsC3498−
mutants under all growth conditions, indicating a slight growth
defect (Fig. 3A). The doubling time of wild-type, OlsA−/H11002
and PlsC316−/H11002 strains that were grown in liquid MPYE medium
were 100, 122, and 131 min, respectively.

Double mutants with all possible combinations of olsA, plsC316, and plsC3498 were then sought to probe any possible
functional redundancy between these genes. Like the single
mutants, the PlsC316/PlsC3498 (SA14) and the OlsA−
PlsC3498− (SA12) double mutants were readily obtained.

FIG. 2. Comparison of various AGPAT homologues of
R. capsulatus. The R. capsulatus (Re) AGPAT homologues were aligned with the
E. coli (Ec) and N. meningitidis (Nm) AGPAT sequences using the program ClustalW and presented using the BOXSHADE, version 3.21, software.
Identical residues are shaded in black, and similar residues are shaded in gray. The catalytic (HX_D) motif (24) and the substrate-binding
(PEGTR) motif of GPATs and AGPATs are boxed and indicated by asterisks.

FIG. 3. Characterization of plsC mutants. (A) Growth of wild-type
(wt), olsA (SA4), plsC316 (SA13), and plsC3498 (SA11) null mutants on
MPYE medium at 35°C under aerobic conditions after 2 days of
incubation. (B) Growth of plsC316 mutant harboring a plasmid with
(SA13/pMRC) or without (SA13/pRK415) olsA under the same
conditions as described for panel A. (C) Comparison of the c-type cyt
profiles of R. capsulatus plsC316 and olsA mutants. Membrane fractions
were isolated from cells grown at 35°C in MPYE medium, pro-
tected, and proteins were separated using 16.5% tricine-SDS-PAGE, and the
the cbb3-Cox (cyt bc1 complex (c0) and the electron carrier cyt c1 (c1) are
indicated on the left together with the 32.5- and 25-kDa molecular size
markers.
These mutants were able to grow on all media tested and exhibited the corresponding single-mutant phenotypes (slow growth and OL deficiency, respectively). Thus, combined inactivation of \( \text{plsC}316 \) with \( \text{plsC}3498 \) or of \( \text{olsA} \) with \( \text{plsC}3498 \) had no deleterious growth effect, indicating that the function of \( \text{plsC}3498 \) was not redundant with either of the other genes. In contrast, despite many attempts under various conditions, inactivation of both \( \text{olsA} \) and \( \text{plsC}316 \) was impossible. The inability to obtain an \( \text{OlsA}^- \) \( \text{PlsC316}^- \) double mutant strongly suggested that an intact copy of either \( \text{olsA} \) or \( \text{plsC}316 \) was required to support growth of \( R. \text{capsulatus} \) under the conditions tested. This observation was further confirmed by using \( \text{olsA} \) or \( \text{plsC316} \) diploid strains (SA15 and SA16) as recipients for interposon mutagenesis (Table 1). These diploid strains carried a copy of a given gene on the chromosome and another copy of the same gene on an autonomously replicating plasmid. Using these strains, mutants carrying inactive chromosomal copies of both \( \text{olsA} \) and \( \text{plsC316} \) but complemented by plasmid-borne copies of either of these genes were readily obtained. The genetic data therefore indicated that an intact copy of either \( \text{plsC}316 \) or \( \text{olsA} \) was required for growth of \( R. \text{capsulatus} \).

The cyt \( c \) profiles and membrane polar lipid and fatty acid compositions of \( R. \text{capsulatus} \) mutants lacking various \( \text{plsC} \) homologues. Considering that \( OL \) and, hence, its biosynthetic genes \( \text{olsA} \) and \( \text{olsB} \) are required for the presence of normal steady-state amounts of several \( c \)-type cyt and \( cbb \)-type cyt activity in \( R. \text{capsulatus} \), we examined the effect of \( \text{plsC}316 \) inactivation on the \( c \)-type cyt content of \( R. \text{capsulatus} \). Analyses of various \( \text{plsC}316 \) (Fig. 3C, lane 3) and also \( \text{plsC3498} \) (data not shown) single or double mutants indicated that, unlike \( \text{OlsA}^- \) mutants, these mutants produced wild-type levels of membrane-bound (Fig. 3C, lane 1) and soluble \( c \)-type cyt and had \( cbb \)-type cyt activities (data not shown).

Total lipid compositions of the \( \text{PlsC316}^- \) and \( \text{PlsC3498}^- \) mutants were next examined after labeling with \( [1-14\text{C}]\)acetate. Using these strains, mutants carrying inactive chromosomal copies of both \( \text{olsA} \) and \( \text{plsC316} \) but complemented by plasmid-borne copies of either of these genes were readily obtained. The genetic data therefore indicated that an intact copy of either \( \text{plsC}316 \) or \( \text{olsA} \) was required for growth of \( R. \text{capsulatus} \). Analyses of various \( \text{plsC}316 \) (Fig. 3C, lane 3) and also \( \text{plsC3498} \) (data not shown) single or double mutants indicated that, unlike \( \text{OlsA}^- \) mutants, these mutants produced wild-type levels of membrane-bound (Fig. 3C, lane 1) and soluble \( c \)-type cyt and had \( cbb \)-type cyt activities (data not shown).

Total lipid compositions of the OL-deficient strains (Fig. 3B). However, an \( \text{OlsA}^- \) mutant carrying an intact copy of \( \text{plsC}316 \) was still devoid of OL.

FIG. 4. Total lipid composition of \( \text{plsC}316 \) and \( \text{plsC3498} \) null mutants of \( R. \text{capsulatus} \). In all cases, total polar lipids were extracted from \( [1-14\text{C}]\)acetate-labeled cells, similar amounts (60,000 cpm) were deposited on TLC plates, and 2D-TLC analyses were carried out as described in Materials and Methods. DGTS, diacylglyceryl trimethylhomoserine; DMPE, phosphatidyl-N,N dimethylethanolamine. The vertical and horizontal arrows at the origin O refer to the first and second dimension of solvent migrations, respectively. The radioactivity associated with each spot was determined and is given in Table 2.

The cyt \( c \) profiles and membrane polar lipid and fatty acid compositions of \( R. \text{capsulatus} \) mutants lacking various \( \text{plsC} \) homologues. Considering that \( OL \) and, hence, its biosynthetic genes \( \text{olsA} \) and \( \text{olsB} \) are required for the presence of normal steady-state amounts of several \( c \)-type cyt and \( cbb \)-type cyt activity in \( R. \text{capsulatus} \). Using these strains, mutants carrying inactive chromosomal copies of both \( \text{olsA} \) and \( \text{plsC316} \) but complemented by plasmid-borne copies of either of these genes were readily obtained. The genetic data therefore indicated that an intact copy of either \( \text{plsC}316 \) or \( \text{olsA} \) was required for growth of \( R. \text{capsulatus} \). Analyses of various \( \text{plsC}316 \) (Fig. 3C, lane 3) and also \( \text{plsC3498} \) (data not shown) single or double mutants indicated that, unlike \( \text{OlsA}^- \) mutants, these mutants produced wild-type levels of membrane-bound (Fig. 3C, lane 1) and soluble \( c \)-type cyt and had \( cbb \)-type cyt activities (data not shown).

Total lipid compositions of the \( \text{PlsC316}^- \) and \( \text{PlsC3498}^- \) mutants were next examined after labeling with \( [1-14\text{C}]\)acetate followed by extraction and 2D-TLC separation, as described in Materials and Methods. The data showed no qualitative differences between the \( \text{PlsC316}^- \) and \( \text{PlsC3498}^- \) mutants and the wild-type parental strain MT1131 (Fig. 4). Quantitation of polar lipids was performed using ImageQuant software (Typhoon 9410) (Table 2). Compared with a wild-type strain, inactivation of \( \text{plsC316} \) decreased the relative amounts of PE and increased those of PG and OL, whereas inactivation of \( \text{olsA} \) mainly abolished OL production. Overproduction of OL in the absence of \( \text{plsC316} \) (about 10% versus 4% of total lipids in its presence) suggested that in this mutant OlsA activity might have increased to sustain sufficient PA production, concomitantly leading to higher OL production. On the other hand, absence of \( \text{plsC3498} \) had no affect on the total lipid composition of \( R. \text{capsulatus} \) (data not shown), again suggesting that it was unrelated to membrane lipid biosynthesis.

Total fatty acid profiles of \( \text{olsA} \) or \( \text{plsC316} \) mutants were also compared with the \( R. \text{capsulatus} \) wild-type strain MT1131 by using fatty acid methyl ester analysis, as described in Materials and Methods. The data showed that the fatty acid composition of the membrane lipids was altered in the \( \text{olsA}^- \) and \( \text{plsC316}^- \) null mutants (Table 2). In comparison with a wild-type strain, inactivation of \( \text{plsC316} \) decreased and increased modestly the relative amounts of saturated C16 and C18 fatty acids, respectively. Moreover, it drastically decreased the amount of unsaturated C16 but not unsaturated C18 fatty acids. On the other hand, inactivation of \( \text{olsA} \) somewhat increased the amounts of saturated, but not unsaturated, C16 and C18 fatty acids compared to a wild-type strain.

Both \( R. \text{capsulatus} \) \( \text{olsA} \) and \( \text{plsC316} \) can complement an \( E. \text{coli} \) \( \text{plsC} \) mutant in vivo. Pronounced similarities observed between various PlsC homologues (Fig. 2) led us to probe whether any of the \( R. \text{capsulatus} \) PlsC homologues could complement the \( E. \text{coli} \) \( \text{plsC} \) mutant, SM2-1, producing a temperature-sensitive AGPAT (12). Plasmid pBAD derivatives, expressing upon induction by l-arabinose either \( \text{olsA} \), \( \text{plsC316} \), or \( \text{plsC3498} \), were constructed as described in Materials and Methods and transformed into the strain SM2-1 at 30°C. Appropriate transformants were tested for their ability to grow at 42°C in the presence of 2% l-arabinose. The plasmid pSEM17 or pSEM25 carrying either \( \text{olsA} \) or \( \text{plsC316} \) was able to complement the \( E. \text{coli} \) \( \text{plsC} \) mutant, SM2-1, for growth at 42°C but only upon induction with l-arabinose (Fig. 5A). Under similar conditions, no complementation was observed with the plasmid pDML1 carrying \( \text{plsC3498} \). Thus, both \( \text{OlsA} \) and \( \text{PlsC316} \), but not \( \text{PlsC3498} \), acted as functional homologues of \( E. \text{coli} \) PlsC and produced apparently temperature-resistant AGPAT activity. Furthermore, it was also noted that \( \text{plsC316} \) provided a more vigorous growth than \( \text{olsA} \). Immunoblot analyses were carried out to confirm that genetic complementation was due to the production in \( E. \text{coli} \) of \( R. \text{capsulatus} \) OlsA or PlsC. As expected, upon induction by l-arabinose, \( \alpha \)-Myo
epitope-tagged proteins with molecular masses of approximately 31 and 29.5 kDa were detected by using anti α-Myc antibodies in the E. coli SM2-1 derivatives harboring OlsA (SM2-1/pSEM17) and PlsC316 (SM2-1/pSEM25), respectively.

Availability of plasmids carrying α-Myc epitope-tagged alleles of OlsA and PlsC316 allowed us to probe whether these proteins were produced in active forms in R. capsulatus. The plasmids pSEM18 and pSEM26 carrying olsA and plsC316, respectively, were coexpressed in SA4 (Δ[oolsA::spe]) and SA13 (Δ[plsC316::kan]). Transconjugants SA4/pSEM18 and SA13/pSEM26 thus obtained were grown in MPYE medium with or without 2% L-arabinose. Immunoblot analyses revealed that they contained proteins of approximately 31 kDa and 29.5 kDa that reacted with anti-Myc antibodies (data not shown). The levels of the proteins produced in R. capsulatus were lower than those seen in E. coli, but the wild-type phenotypes of the transconjugants in respect to OL, c-type cyt production, and better growth indicated that the epitope-tagged versions of OlsA and PlsC316 were functional.

The E. coli plsB and plsC gene products, conferring GPAT and AGPAT activities, share partial amino acid sequence homologies and are thought to function coordinately (Fig. 1A) (13). Considering that some acyltransferases, like the Clostridium butyricum plsD exhibiting functional GPAT activity, can complement an E. coli PlsB− mutant (23) and that plsC3498 showed similarity to plsD (20% identity and 34% similarity), we used the E. coli mutant SJ22 to investigate whether olsA, plsC316, or plsC3498 exhibited functional GPAT activity. This mutant carries both the plsB26 and plsX50 mutations and requires supplementation with G3P for growth (39). Upon transformation of the plasmids pSEM17 (olsA), pSEM25 (plsC316), and pDML1 (plsC3498) into SJ22, no complementation for G3P auxotrophy was observed, indicating that none of these genes produced GPAT activity and especially that plsC3498 was not a homologue of plsB in R. capsulatus.

R. capsulatus OlsA and PlsC316 exhibit AGPAT activities and synthesize PA in vitro. In an attempt to define the biochemical function(s) of OlsA and PlsC316, combined GPAT-AGPAT activities were assayed in vitro by using radioabeled G3P as the acyl acceptor and acyl-ACP as the acyl donor, as described in Materials and Methods. Unlike the E. coli GPAT and AGPAT enzymes, which can use either acyl-CoA or acyl-ACP as acyl donors (47), Rhodobacter sphaeroides enzymes exhibit high specificity for acyl-ACP compared to acyl-CoA (34). No significant enzyme activity was observed with the acyl-CoA substrate in R. capsulatus (data not shown) as in R. sphaeroides. Considering that R. capsulatus lipids contain predominantly cis-vaccenic acid (cis-11-18:1) fatty acid, cis-vaccn-yl-ACP was prepared as the acyl donor. Purified membrane particles (see the supplemental material) from E. coli plsC(Ts) mutant SM2-1 derivatives harboring olsA or plsC316 and grown at 42°C in the presence of L-arabinose were assayed. Time course assays monitoring the production of radiolabeled LPA and PA were carried out as described in Materials and Methods. Control experiments established that the activities measured were vaccenyl-ACP and membrane particle dependent (data not shown), and the endogenous activity detected using membranes from SM2-1 cells grown at 30°C and subsequently incubated at 42°C was very low. The data obtained revealed that membranes from SM2-1 derivatives producing either OlsA or PlsC316 exhibited measurable amounts of combined GPAT-AGPAT activity (Fig. 6A). Moreover, PlsC316-containing membrane particles displayed much higher specific activities than either those containing OlsA or those from SM2-1 cells grown at 30°C.

As the combined GPAT-AGPAT assay using radioactive G3P reflects the production of both LPA and PA, separate formation of LPA via GPAT and of PA via AGPAT activities was also determined. Products of a similar enzymatic reaction were analyzed by 1D-TLC, and LPA and PA were identified by comparison of their Rf values with those of standard markers.
FIG. 6. GPAT-AGPAT activities exhibited by appropriate E. coli plsC(Ts) mutants harboring R. capsulatus plsC homologues as well as R. capsulatus wild-type, olsA, and plsC316 mutants. (A) Time course assays of GPAT-AGPAT activities in E. coli plsC mutant harboring olsA or plsC316 were performed using radioactive G3P, vaccenyl-ACP, and membrane particles (prepared as described in the supplemental material) from SM2-1 cells grown at 30°C (SM2-1), SM2-1 cells grown at 30°C with a subsequent 30-min incubation at 42°C (SM2-1*), SM2-1 cells harboring olsA, and SM2-1 cells harboring plsC316, as described in Materials and Methods. The data shown are the means of two independent experiments with the standard errors, as indicated. (B) Assays similar to those shown in panel A were performed at 35°C for 5 min, and labeled lipids (approximately 7,000 cpm total) were extracted and separated by 1D-TLC, as described in Materials and Methods. LPA and PA produced using membranes from SM2-1 cells grown at 30°C (lane 1), SM2-1 grown at 30°C with a subsequent 30-min incubation at 42°C (lane 2), SM2-1 cells harboring olsA (lane 3), and SM2-1 cells harboring plsC316 (lane 4) are shown. Note the absence of PA production in lane 2 and PA overproduction in lane 4. (C) Time course assays of GPAT-AGPAT activities in wild-type (wt), ΔolsC (SA4), and Δplsc316 (SA13) strains were performed as described for panel A. The data shown are the means of two independent experiments with the standard errors as indicated. (D) Labeled lipids (approximately 2,000 cpm total) were prepared and separated by 1D-TLC, as described for panel B. Note that the PA produced using membranes from the wild-type strain MT1131 and the ΔolsA mutant are readily seen while that produced by the Δplsc316 mutant is barely detectable.

As expected, all membrane particles produced some amounts of LPA, which reflected the intact GPAT activity of the E. coli host SM2-1. On the other hand, membrane particles from heat-treated (42°C for 30 min) SM2-1 cells (grown at 30°C) did not produce any (Fig. 6B, lane 2), whereas those from non-heat-treated cells produced detectable amounts of LPA. Similarly, E. coli SM2-1 derivatives harboring the R. capsulatus olsA or plsC316 contained AGPAT activity even when grown at 42°C. Moreover, membrane particles harboring PlsC316 or OlsA produced visibly more PA than their parent SM2-1 grown at 30°C (Fig. 6B, lanes 1, 3, and 4). Quantitative estimations using ImageQuant software indicated that the PA production rate was highest (approximately 10 pmol/min/μg of membrane protein) in SM2-1 cells with PlsC316, followed by cells with OlsA (0.875 pmol/min/μg of membrane protein), and lowest in SM2-1 cells grown at 30°C (0.34 pmol/min/μg of membrane protein). Apparently, expression of OlsA or PlsC316 yielded, respectively, approximately 2.5- or 11-fold more PA production than the endogenous activity present in the E. coli plsC(Ts) mutant SM2-1 grown at 30°C. We therefore concluded that both R. capsulatus olsA and plsC316 gene products have AGPAT activities, which explained why the presence of either gene was sufficient for membrane glycerophospholipid production and growth of this species. In addition, the vigorous AGPAT activity and the inability to produce OL distinguished PlsC316 from the bifunctional OlsA involved in both PA and OL synthesis and suggested that PlsC316 might be the major enzyme responsible for PA biosynthesis in R. capsulatus.

AGPAT activities of R. capsulatus PlsC316− or OlsA− mutants. Combined GPAT-AGPAT activities in vitro were also determined using membrane preparations from R. capsulatus OlsA− (SA4) or PlsC316− (SA13) mutants to further establish that PlsC316 is the main enzyme carrying out PA biosynthesis in this species. As expected, the OlsA− mutant exhibited a combined GPAT-AGPAT activity that was approximately the same as that seen with the wild-type strain MT1131, and the PlsC316− mutant exhibited much lower (four- to fivefold) GPAT-AGPAT activity relative to both the wild-type strain MT1131 and the OlsA− mutant SA4 (Fig. 6C). Moreover, TLC with quantitative estimations using ImageQuant software showed that the PA production rate in the OlsA− mutant was almost identical (approximately 0.8 pmol/min/μg of membrane protein) to that seen with the wild-type strain MT1131 (Fig. 6D, lanes 1 and 2), whereas the PlsC316− mutant produced barely detectable amounts of PA in vitro (Fig. 6D, lane 3), in agreement with the GPAT-AGPAT activities measured. Therefore, in R. capsulatus PlsC316 is apparently the main AGPAT enzyme producing PA for membrane glycerophospholipid synthesis.

DISCUSSION

At the outset of this work, the genes encoding GPAT and AGPAT enzymes were unidentified experimentally in Rhodobacter species. Our previous studies on c-type cyt biogenesis led us to the identification of the OL biosynthesis genes, olsA and olsB, of R. capsulatus (3) and indicated that the identity of the gene carrying out PA biosynthesis was unclear. The evidence that OlsA− mutants still produced quasi-normal amounts of PA and glycerophospholipids and the occurrence of at least two additional PlsC homologues on the R. capsulatus genome led us to investigate the gene responsible for the AGPAT activity dedicated to PA biosynthesis.

The data obtained in this work indicated that R. capsulatus plsC3498 is not involved in either PA or OL synthesis.
PlsC3498 shares similarity with both NlaA (15% identity and ~25% similarity) and NlaB (~13% identity and ~28% similarity) from *N. meningitidis*. It possesses the HX₄D sequence thought to correspond to the catalytic motif of GPATs and AGPATs, but compared to OlsA and PlsC316, the substrate binding motif (PEGTR) of AGPATs is not conserved (Fig. 2). It has homology to the *C. butyricum* PlsD (20% identity and 34% similarity), but, unlike PlsD (23), it cannot complement a GPAT-less *E. coli* mutant and does not appear to be a functional homologue of PlsB. Thus, the role of *plsc3498* in *R. capsulatus* remains unknown. Moreover, whether *R. capsulatus* has a true PlsB homologue or whether it utilizes exclusively the PlsX/PlsY pathway for LPA biosynthesis (33) awaits the study of *R. capsulatus* ORFs RRC01510 and RRC02960, which exhibit significant homologies to PlsX (pfam02504/COG0416) (10) and PlsY (pfam02660/COG0344), respectively.

A major outcome of this work were the findings that the gene products of both *olsA* and *plsc316* have AGPAT activities and that *R. capsulatus*, unlike *E. coli*, possesses two AGPAT isozymes capable of producing PA. The AGPAT activities of OlsA and PlsC316 were demonstrated by their ability to complement an *E. coli* mutant that has a temperature-sensitive PlsC and by GPAT-AGPAT activity assays in vitro using membrane particles prepared from appropriate *E. coli* and *R. capsulatus* strains. It was noted that PlsC316 conferred higher AGPAT activities than OlsA but displayed no OL synthesis activity at least in vivo, as OlsA⁻ mutants are devoid of OL. Moreover, PlsC316⁻ mutation had no effect on the steady-state amounts of c-type cyt, consistent with their OL contents. Thus, our overall findings suggested that PlsC316 is the major AGPAT enzyme, dedicated to PA biosynthesis only. This finding was further supported by the fact that *R. capsulatus* PlsC316⁻ mutants have much lower AGPAT activities than OlsA⁻ mutants. On the other hand, OlsA is primarily responsible for OL biosynthesis and also produces some PA to sustain slower growth of *R. capsulatus*. Although OlsA and PlsC316 share homologies with *E. coli* PlsC and as AGPAT isoforms, they have distinct but overlapping cellular functions. Finally, as double mutants lacking both of these enzymes are lethal, no other gene encoding another functional AGPAT enzyme appears to be present in the *R. capsulatus* genome.

The O-acyltransferase OlsA is able to recognize both lysornithine lipid ([LOL] a long-chain acyl amide of ornithine) and LPA (esterified sn-G3P) as substrates to which it transfers an acyl group from an acyl-ACP to yield OL and PA, respectively. In both cases, the reaction catalyzed is esterification of an α-CHOH moiety, suggesting broad substrate specificity for this enzyme beyond the accepting group. However, this relaxed substrate recognition does not seem to be a general property of all OlsA enzymes. Apparently, homologues of OlsA from some other bacteria, e.g., *Sinorhizobium meliloti* (48) and *P. fluorescens* (14), do not display any AGPAT activity, as indicated by their inability to complement an *E. coli plsc(T)s* mutant, unlike the *R. capsulatus* OlsA. Although OlsA enzymes from different species show pronounced similarities to AGPATs of prokaryotes and eukaryotes and contain two conserved domains and the consensus (HX₄D) catalytic motif, it is unclear why some of them are bifunctional and can produce both OL and PA while others can synthesize only OL. A possibility is that different OlsA enzymes might have differing specificities for their acyl donor substrates (acyl-ACP) rather than acyl acceptor substrates (LOL and LPA). If this is the case, then the *R. capsulatus* but not the *S. meliloti* or *P. fluorescens* OlsA seems to recognize *E. coli* ACP efficiently. Also consistent with the more selective behavior of *S. meliloti* OlsA is our earlier observation that *S. meliloti* OlsA mutants can be complemented with *R. capsulatus* OlsA but not vice versa (3), suggesting that the latter enzyme has a more relaxed ACP specificity to recognize *S. meliloti* ACP for OL synthesis.

Why some organisms have multiple AGPAT isozymes is interesting. In eukaryotes, the fact that AGPATs are involved in different regulatory circuits with different substrate preferences, like cellular responses to cytokines and growth factors, has been suggested as an explanation the occurrence of multiple AGPATs expressed in different tissues (9, 20, 32). Similarly, some bacterial species including *N. meningitidis*, *N. gonorrhoeae*, and *P. fluorescens* have multiple AGPATs, whereas others, like *E. coli*, appear to have only one such enzyme. It has been suggested that the different isozymes might play different roles, such as fine-tuning the membrane lipid and fatty acid profiles in diverse environments (14, 42, 45). Indeed, while *P. fluorescens* OlsA⁻ mutants exhibited no major changes in the membrane phospholipid and fatty acid profiles, inactivation of *P. fluorescens* AGPAT isozymes PatB and HdtS did alter the fatty acid profile of phospholipids and some membrane properties (14), as seen here with *R. capsulatus* OlsA⁻ and PlsC⁻ mutants.

In the case of *N. meningitidis*, apparently both NlaA and NlaB proteins displayed AGPAT activity in vitro as they complemented a temperature-sensitive *E. coli plsc(T)s* mutant. Furthermore, this species might have at least an additional enzyme with AGPAT activity as an NlaA⁻ NlaB⁻ double mutant is viable and has AGPAT activity (42, 45). Indeed, *R. capsulatus* OlsA and PlsC316 show noteworthy similarities to NlaA (OlsA, ~21% identity and ~32% similarity; PlsC316, ~16% identity and ~30% similarity) and NlaB (OlsA, ~16% identity and 27% similarity; PlsC316, ~26% identity and ~32% similarity), as depicted in Fig. 2. But a closer examination suggests that NlbA seems to be more homologous to PlsC316 and NlaA to OlsA, especially based on the pfam01553/COG0204 motif, suggesting that *N. meningitidis* might contain OL.

In summary, this work demonstrated that of the three *plsc* homologues encountered in the *R. capsulatus* genome, *plsc3498* is not involved in membrane phospholipids or OL biosynthesis. On the other hand, *olsA* and *plsc316* encode efficient AGPAT enzymes able to sustain membrane glycerophospholipid synthesis and growth of *R. capsulatus*; of the two isoenzymes, PlsC316 seems to be the major enzyme responsible for PA biosynthesis. Finally, the finding that *R. capsulatus* OlsA produces both OL and PA demonstrated for the first time that some OlsA homologues are bifunctional enzymes with overlapping activities. Future studies may shed light on why nature has evolved and conserved multifunctional AGPAT enzymes and how organisms use the specificity and control the promiscuity of these isoenzymes in response to their changing environments.
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