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Isolation and Expression of the *Rhodobacter sphaeroides* Gene (*pgsA*) Encoding Phosphatidylglycerophosphate Synthase

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The *Rhodobacter sphaeroides pgsA* gene ($pgsA_{Rs}$), encoding phosphatidylglycerophosphate synthase ($PgsA_{Rs}$), was cloned, sequenced, and expressed in both *R. sphaeroides* and *Escherichia coli*. As in *E. coli*, $pgsA_{Rs}$ is located immediately downstream of the *uvrC* gene. Comparison of the deduced amino acid sequences revealed 41% identity and 69% similarity to the *pgsA* gene of *E. coli*, with similar homology to the products of the putative *pgsA* genes of several other bacteria. Comparison of the amino acid sequences of a number of enzymes involved in CDP-diacylglycerol-dependent phosphatidyltransfer identified a highly conserved region also found in $PgsA_{Rs}$. The $pgsA_{Rs}$ gene carried on multicopy plasmids was expressed in *R. sphaeroides* under the direction of its own promoter, the *R. sphaeroides rrnB* promoter, and the *E. coli lac* promoter, and this resulted in significant overproduction of $PgsA_{Rs}$ activity. Expression of $PgsA_{Rs}$ activity in *E. coli* occurred only with the *E. coli lac* promoter. $PgsA_{Rs}$ could functionally replace the *E. coli* enzyme in both a point mutant and a null mutant of *E. coli pgsA*. Overexpression of $PgsA_{Rs}$ in either *E. coli* or *R. sphaeroides* did not have dramatic effects on the phospholipid composition of the cells, suggesting regulation of the activity of this enzyme in both organisms.

Rhodobacter sphaeroides is a gram-negative bacterium capable of growth by aerobic and anaerobic respiration, fermentation, and anoxygenic photosynthesis (34). *R. sphaeroides* responds to changes in its environment with both physiological and morphological adaptations. Under aerobic conditions, the organism has a typical gram-negative outer membrane and cytoplasmic membrane. At low oxygen tension a dramatic differentiation of the cytoplasmic membrane occurs, with the formation of an intracytoplasmic membrane. The intracytoplasmic membrane is physically continuous with the cytoplasmic membrane but structurally and functionally distinct. The intracytoplasmic membrane contains all of the components necessary for photosynthesis (34). Studies on the regulation of intracytoplasmic membrane assembly have demonstrated the cell cycle-specific insertion of phospholipids into the intracytoplasmic membrane of photoheterotrophically growing cells (13, 32), with protein and photopigments incorporated continuously into the intracytoplasmic membrane throughout the cell cycle. Insertion of phospholipids into the intracytoplasmic membrane is concurrent with the onset of cell division and is the result of the net transfer of phospholipids previously synthesized outside the intracytoplasmic membrane (13, 32, 51). Although the intracytoplasmic membrane and its protein components have been extensively studied, the role specific phospholipid species and general phospholipid metabolism play in the induction, synthesis, assembly, and function of the intracytoplasmic membrane has received only limited attention, mainly because of the lack of detailed biochemical information relating to phospholipid metabolism and lack of genetic information on the synthesis of specific phospholipid species in this organism.

The major phospholipids of *R. sphaeroides* are phosphatidylethanolamine (40%), phosphatidylcholine (18%), and phosphatidylglycerol (27%) (1). About 3% of the lipids in this organism are sulfolipids, which are also found in larger amounts in plants (1). Mutants defective in sulfolipid synthesis have no known phenotype, indicating no apparent requirement for intracytoplasmic membrane function (3). The presence of phosphatidylcholine, which is not present in other gram-negative bacteria such as *Escherichia coli*, might have suggested that this phospholipid is important for photoheterotrophic growth, but mutants almost completely devoid of phosphatidylcholine have no apparent phenotype (1). However, it is most probable that changes in the levels of the other major phospholipids, phosphatidylethanolamine (which is the precursor to phosphatidylcholine) and phosphatidylglycerol, would have dramatic effects on both the photo- and chemoheterotrophic growth properties of this organism as well as the induction, assembly, and functioning of the intracytoplasmic membrane. Alterations in the steady-state level of phosphatidylethanolamine (4, 10, 25, 43, 56) and phosphatidylglycerol (37, 40, 62, 64) in *E. coli* have resulted in major effects on cell physiology which have been related to the specific functions of these phospholipids in important cellular processes.

As a first step in the analysis of the role of specific phospholipids in *R. sphaeroides* cell function, the genes involved in the synthesis of the major phospholipid species must be identified and mutants with alterations in these genes must be constructed and analyzed. Previous work has confirmed that phospholipid biosynthesis in *R. sphaeroides* is similar to that of *E. coli* (6, 7). Synthesis occurs in the cytoplasmic membrane, and there is no de novo synthesis of phospholipids in the intracytoplasmic membrane (5). Thus, by using standard molecular genetic and biochemical techniques, it should be possible to isolate and characterize phospholipid biosynthetic genes in *R. sphaeroides* by utilizing the large genetic and biochemical database available for *E. coli*. Using this approach, we have identified, cloned, and sequenced the gene ($pgsA_{Rs}$) encoding phosphatidylglycerophosphate synthase ($PgsA_{Rs}$), which catalyzes the committed step of phosphatidylglycerol biosynthesis in both *R. sphaeroides* and *E. coli* (52, 54), and have begun to

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
R. sphaeroides 2.4.1	Wild type	63
E. coli		
DH5α	ϕ80d*lac*ZΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44*λ^- *gyrA96 relA1*	55
EH150	*purA psd*(Ts)	25
HB101	*mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*(Sm^r) *supE44* λ^-	58
HD30	*pgsA*::Km In(*rrnD-rrnE*)1	26
MN7	*pgsA444 lpxB*(Ts) *strA nalA his thr*	46
S17-1	RP4-2-Tc::Mu-Km::Tn7 Sm^r Tp^r *pro hsdR hsdM*$^+$	60
Plasmids		
pBBR1MCS-2	Cloning vector, Km^r	36
pBluescriptIIKS	Cloning vector, Ap^r	Stratagene
pRK415	Cloning vector, Tc^r	33
pHP45	Ap^r Sm^r/Spc^r Ω cartridge	50
pUC195B	Ap^r $rrnB_{Rs}$	19
pLAB14	20 to 25 kb of *R. sphaeroides* DNA in cosmid pLA2917	18
pBSP8	pBluescript with a 1.4-kb *Pst*I fragment of pLAB14	This work
pB1	pBBR1MCS-2 with a 1.4-kb *Pst*I fragment of pLAB14	This work
pBrrn	pBBR1MCS-2 with $rrnB_{Rs}$ (273-bp *Hin*dIII-*Bam*HI fragment) of pUC195B	This work
pB2	pBrrn with $pgsA_{Rs}$ (793-bp *Sac*II-*Sac*I fragment) of pB1	This work
pB3	pBBR1MCS-2 with $pgsA_{Rs}$ (793-bp *Sac*II-*Sac*I fragment) of pB1	This work
pB3Ω	pB3 with Sm^r/Spc^r cartridge from pHP45 inserted at the *Sac*I site	This work
pHD102	Cm^r *pgsA*$^+$ *ori*(Ts)	26
pRK1	pRK415 with a 1.4-kb *Pst*I fragment of pLAB14	This work

characterize the functional properties of the *R. sphaeroides* enzyme in both organisms.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are described in Table 1. *E. coli* strains, including MN7/pB3Ω and HD30/pB3Ω, were grown aerobically at 37°C in Luria-Bertani (LB) medium (liquid or agar) (42) except for the temperature-sensitive strains MN7 and HD30/pHD102, which were grown at 30°C. Strain MN7 carries a *lpxB*(Ts) allele which is lethal at the restrictive temperature in a strain carrying a mutation (*pgsA444*) which results in a reduced activity for the PgsA (46). Strain HD30 carries a null allele of the essential $pgsA_{Ec}$ gene and therefore requires a plasmid copy of the gene which in the case of plasmid pHD102 is carried on a vector that is itself temperature sensitive for replication (26). *R. sphaeroides* 2.4.1 was grown aerobically at 30°C in Sistrom's minimal medium A (liquid or agar plates) containing 0.4% succinate (12). When appropriate, tetracycline (1 μg/ml for *R. sphaeroides* and 10 μg/ml for *E. coli*), kanamycin (25 μg/ml), ampicillin (50 μg/ml), streptomycin (50 μg/ml), spectinomycin (50 μg/ml), and/or chloramphenicol (25 μg/ml) was added to the growth media. Plasmids pRK415 (33) and pBBR1MCS-2 (36) and their derivatives were introduced into *R. sphaeroides* by diparental matings using the *E. coli* host strain S17-1 (60) or by triparental matings involving the donor *E. coli* strain HB101 harboring the helper plasmid pRK2013 (20). Exconjugants were selected by utilizing tellurite in the medium as previously described (44).

Complementation of strains MN7 and HD30. After the introduction of plasmid pB3Ω into strain MN7 by transformation, cells were plated at 30°C and incubated overnight. Individual colonies were picked into LB medium, and serial dilutions were made and plated in duplicate at 42 and 30°C. After overnight growth viable counts were done, and the numbers of colonies found at 30 and 42°C were compared. Positive complementation was scored if the same numbers of colonies were found on plates grown under permissive and nonpermissive conditions.

After transformation of strain HD30/pHD102 with plasmid pB3Ω and growth on plates overnight at 30°C, individual colonies were isolated and grown overnight in LB medium plus antibiotics (kanamycin, streptomycin, and spectinomycin) and IPTG (isopropyl-β-D-thiogalactopyranoside) at 30°C. The overnight culture was diluted into LB medium plus antibiotics, grown aerobically at 30°C for 30 min, and then shifted to 42°C for 4 h in order to cure strain HD30 of plasmid pHD102, which is temperature sensitive for replication. After 4 h, cells were plated on LB agar plates plus antibiotics, and duplicate sets were incubated overnight at 30 and 42°C. Resultant colonies surviving at 42°C were checked for Cm^s to ensure the loss of plasmid pHD102 (26).

DNA isolation and manipulation. Plasmids were isolated as previously described (45). Transformations and other DNA manipulations are described elsewhere (58). Southern and colony hybridizations were performed as described elsewhere (58). Probes were labeled by using the Genius DNA labeling and detection kit (Boehringer Mannheim). Results were visualized with Lumi-Phos (Boehringer Mannheim). PCRs were performed by following the manufacturer's instructions and using *Taq* polymerase (Promega). A primer (5′-TTCAGGAC GCTACTTGTGTA-3′) complementary to the end of the IS*50* segment of plasmid pBS492 (41) and the T7 primer (Promega) were used in a reaction with plasmid pBS492 in order to isolate a DNA fragment carrying the potential $pgsA_{Rs}$ gene. A Perkin-Elmer Cetus thermal cycler was used, and conditions were the following: 7 min of incubation at 95°C, followed by 30 cycles of 95°C for 1 min, 50°C for 2 min, and 75°C for 1 min. The PCR products were gel purified and purified with GeneClean (Bio 101).

Construction of expression plasmids. The *Pst*I fragment from plasmid pBSP8 (Fig. 1A) was cloned into plasmids pRK415 and pBBR1MCS-2 to yield the plasmids shown in Fig. 1B and C. The $rrnB_{Rs}$ promoter, isolated as a *Hin*dIII-*Bam*HI fragment from plasmid pUC195B (19), was subcloned into plasmid pBBR1MCS-2 to give plasmid pBrrn. Plasmid pB2 (Fig. 1B) was constructed by cloning the *Sac*I-*Sac*II fragment of plasmid pB1 (containing $pgsA_{Rs}$) between the *Sac*I and *Sac*II sites of plasmid pBrrn. The *Sac*I-*Sac*II insert was also ligated into *Sac*I-*Sac*II-digested plasmid pBBR1MCS-2 to create plasmid pB3 (Fig. 1B), in which the $pgsA_{Rs}$ gene is fused to the lac_{Ec} promoter. Plasmid pB3Ω (Fig. 1C) was constructed by insertion of a Sm^r/Spc^r cartridge (50) into the *Sac*I site of plasmid pB3 using an isoschizomer to *Sac*I, *Ecl*136II, which creates blunt ends and is thus compatible with the *Sma*I sites flanking the Sm^r/Spc^r cartridge.

Detection of PgsA activity. Overnight cultures (5 ml each) of either *R. sphaeroides* or *E. coli* were pelleted and resuspended in 0.4 ml of 0.1 M Tris-HCl, pH 7.0, and sonicated on ice for 20 s three times. The cell debris was removed by a 1-min low-speed spin. Enzyme specific activity (expressed in nanomoles per minute per milligram of protein) was assayed at 37°C by monitoring the CDP-diacylglycerol-dependent incorporation of L-[2-^{3}H]glycerol-3-phosphate into chloroform-soluble material as described previously (26), except that cell lysates were incubated at 50°C for 20 min prior to the assay to inactivate the temperature-sensitive $pgsA_{Ec}$ gene product of strain MN7 (46). Wild-type PgsA activity has been shown to be stable to treatment at 50°C in both organisms (17, 46). The subcellular location of PgsA activity was determined after centrifugation of cell lysates at 100,000 × *g* for 45 min. The membrane fraction (pellet) was resuspended in 0.1 M Tris-HCl, pH 7.0. The soluble and membrane fractions were assayed as described above. Protein concentrations were measured by using the bicinchoninic acid protein reagent (Pierce Chemical Co.).

Phospholipid composition. The phospholipid composition of both *R. sphaeroides* and *E. coli* strains was determined as described elsewhere (64), except that strains were labeled with 10 μCi of $^{32}PO_4$ (Amersham) per ml and grown for at least five generations prior to the harvesting of cells in stationary phase. Some cultures were supplemented with arbutin as previously described (31). The phospholipids from *E. coli* were separated by one-dimensional thin-layer chromatography with boric acid-impregnated silica gel plates as described by Fine and Sprecher (21). For *R. sphaeroides* the two-dimensional thin-layer chromatography system of Poorthius et al. (49) was used to separate phosphatidylcholine from phosphatidylglycerol. Radioactivity was quantified with a Betagen; the mole percent of each phospholipid was calculated on the basis of its phosphate content.

DNA sequencing. Plasmid DNA was purified by using the Wizard 373 kit (Promega), and reactions were performed by the *Taq* Dye-deoxy Terminator (Applied Biosystems) method and run on an Applied Biosystems Sequenator. All plasmids were derivatives of pBluescriptKS (Stratagene) and were sequenced by using the T7 and T3 primers. Sequence assembly was carried out with the Geneworks program (Intelligenetics Inc.) and the Genetics Computer Group software package (11). Sequence files were analyzed and compared with protein and DNA databases by using the BLAST (23) server at the National Center for Biotechnology Information (Bethesda, Md.) as well as the Genetics Computer Group software package.

Nucleotide sequence accession number. The complete DNA sequence (derived from both strands) of the *Pst*I fragment can be obtained from GenBank (accession number U29587); several errors reported in the initial sequence for part of this region (41) have been corrected.

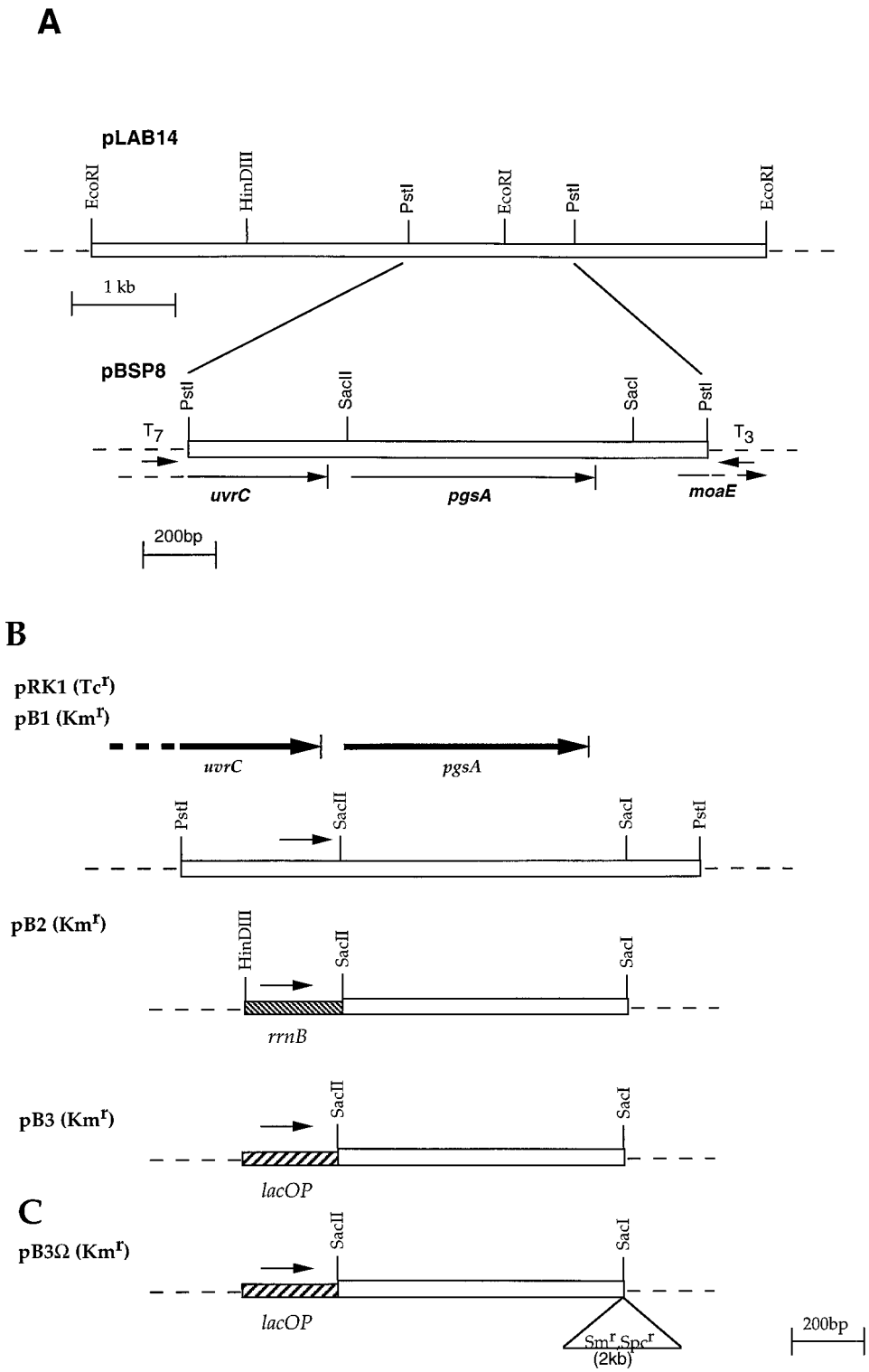

FIG. 1. Partial restriction map of the region of cosmid DNA containing the $pgsA_{Rs}$ gene and construction of $pgsA_{Rs}$ expression plasmids. (A) Approximately 6.5 kb of the 25-kb *R. sphaeroides* DNA insert in cosmid pLAB14 is shown. A 1.45-kb *Pst*I DNA fragment was subcloned into pBluescript (pBSP8) for DNA sequencing. The location of the $pgsA_{Rs}$ gene, the position of the T7 and T3 promoters, the 5′ portion of the putative *moaE* gene, the 3′ portion of the $uvrC_{Rs}$ gene, and selected restriction sites are shown. Dotted lines denote the vector, and the arrows show the direction of the promoters. (B) Expression plasmids constructed for use in *R. sphaeroides*. (C) Expression plasmid constructed for use in *E. coli* strains. Construction of all plasmids is described in Materials and Methods. Only selected restriction sites are shown. The locations of the *pgsA* and *uvrC* genes and the *rrnB* and *lacOP* promoters are indicated, and the arrows show the direction of the promoters.

RESULTS

Cloning of the *pgsA* gene from *R. sphaeroides* 2.4.1. Other work (41) identified a plasmid, pBS492, which potentially contained a portion of the *R. sphaeroides* homolog to the $pgsA_{Ec}$ gene which encodes PgsA (24, 61). Primers complementary to the vector-insert junction were used in a PCR with pBS492 to generate an 879-bp product. This fragment was isolated and used to probe an *R. sphaeroides* cosmid library (18). Three cosmids which yielded positive signals when probed with the PCR fragment were identified. Cosmid pLAB14 yielded the strongest signal and was further analyzed. A 1.4-kb *Pst*I fragment, which hybridized with the PCR probe, was isolated from pLAB14 and subcloned into pBluescript (pBSP8) (Fig. 1A). DNA sequencing using the T7 primer confirmed that the 879-bp DNA fragment was contained within the *Pst*I insert. The two other cosmids yielding positive signals contained sequences which partially overlapped with the insert in cosmid pLAB14.

DNA sequence of plasmid pBSP8. Plasmid pBSP8 was digested with a variety of restriction enzymes to make a minilibrary of the original *Pst*I insert in the pBluescript vector. The DNA inserts in this minilibrary were sequenced as described in Materials and Methods.

DNA sequence analysis identified the $pgsA_{Rs}$ gene as a complete open reading frame (ORF) and a second, partial ORF encoding 129 amino acids 5′ to the $pgsA_{Rs}$ gene. The partial ORF, encoding 129 amino acids, showed 38% identity and 58% similarity to the *uvrC* gene from *E. coli* and 40% identity and 65% similarity to the *uvrC* gene from *Pseudomonas fluorescens* (data not shown). BLAST searches indicated that the complete ORF (encoding 227 amino acids with a predicted molecular mass of 25,258 Da) was 41% identical and 69% similar to the *pgsA* gene of *E. coli* and 44% identical and 64% similar to the putative *pgsA* gene of *P. fluorescens* (Fig. 2). Similar homology to several other putative bacterial *pgsA* gene products, as shown in Fig. 2A, was also observed. A hydropathy plot (Fig. 3A) suggests that the $PgsA_{Rs}$ is a highly hydrophobic protein. The plot is very similar to that of *E. coli* for the amino-terminal half of the protein (Fig. 3B), but it diverges at the carboxy terminus because of the additional 38 amino acids (residues 149 to 187) found in the $PgsA_{Rs}$ sequence (Fig. 2A). This region contains a potential hydrophobic domain that is lacking in other PgsA homologs, which may indicate a specialized role for this domain in *R. sphaeroides*. Both $PgsA_{Rs}$ and $PgsA_{Ec}$ have been localized to the cytoplasmic membrane of their respective organisms (6, 28, 52). By using the MOTIF search program (2) to compare the $PgsA_{Rs}$ with the data library, a region was identified as containing the CDP-alcohol/phosphatidyltransferase signature (29). When an alignment of seven CDP-diacylglycerol-dependent phospholipid biosynthetic enzymes from several sources (8, 14, 53) was performed, a region of homology which could be of importance in the function of these proteins was identified (Fig. 2B).

No genes encoding tRNAs, such as those found in *E. coli* (24), were found downstream of the $pgsA_{Rs}$ gene. However, an additional ORF bearing 59% identity and 79% similarity to the *moaE* gene (encoding a protein involved in molybdopterin biosynthesis) of *E. coli* (57) was identified. The $uvrC_{Rs}$ ORF is in a different reading frame from the $pgsA_{Rs}$ gene and ends 81 bp before the first of two potential methionine start codons of the $pgsA_{Rs}$ ORF. There are five amino acids between the two methionines, and no potential ribosomal binding site has been identified in front of either methionine codon; thus, the precise start codon of the $pgsA_{Rs}$ is still uncertain.

Analysis of DNA sequences upstream of the $pgsA_{Rs}$ ORF did not reveal any potential σ^{70} promoter sequences. Comparison with the rRNA promoters of *R. sphaeroides* (19) also did not yield any similarities. The 5′ DNA sequences of $pgsA_{Rs}$ and three other *R. sphaeroides* genes were aligned (Fig. 4), and this revealed large sections of homology which may be of significance as either promoter or upstream regulatory regions.

Overexpression of $PgsA_{Rs}$ in *R. sphaeroides*. The *Pst*I insert in plasmid pBSP8 (Fig. 1A) was cloned into the shuttle vectors pRK415 (33) and pBBR1MCS2 (36), which are stably maintained in *R. sphaeroides*. Plasmids pB1 and pRK1 (Fig. 1B) contain the entire 1.4-kb *Pst*I insert; thus, these two plasmids should have the native $pgsA_{Rs}$ gene promoter region. Plasmid pB2 (Fig. 1B) contains the coding region of the $pgsA_{Rs}$ gene fused to the $rrnB_{Rs}$ promoter (19). The fusion junction is at a *Sac*II site 12 bp upstream from the first ATG codon. Plasmid pB3 is similar to plasmid pB2, except that the lac_{Ec} promoter is fused to the $pgsA_{Rs}$ ORF, again at the *Sac*II site. The plasmids were sequenced through the junctions to confirm that they were correct. These expression plasmids, as well as vector controls, were introduced into *R. sphaeroides*, and PgsA activity was assayed as described in Materials and Methods.

Enzyme activity in *R. sphaeroides* containing plasmid pRK1, pB1, or pB2 was at least sevenfold higher than the levels in the control strain containing vector alone (Table 2). The replacement of the native $pgsA_{Rs}$ promoter with the $rrnB_{Rs}$ promoter did not have any effect on the overexpression of enzymatic activity, nor was there any difference among the expression levels when the gene was carried on plasmid pRK1, pB1, or pB2. *R. sphaeroides* harboring plasmid pB3, which contains the $pgsA_{Rs}$ gene fused to the lac_{Ec} promoter yielded only a fourfold increase in enzymatic activity. The subcellular location of the overexpressed protein was determined. As in *E. coli*, more than 95% of the enzyme activity was found in the membrane fraction (data not shown).

Expression of $PgsA_{Rs}$ in *E. coli*. Introduction of plasmid pRK1, pB1, or pB2 into *E. coli pgsA* mutant strains MN7 or HD30 did not rescue the temperature-sensitive phenotype of either strain. Since plasmid pB3 has the $pgsA_{Rs}$ gene under the control of the lac_{Ec} promoter, it was modified to yield plasmid pB3Ω (Fig. 1C) so that it could be selected for in *E. coli pgsA* mutants by using Spc^r/Sm^r. Plasmid pB3Ω was introduced into both strains MN7 and HD30 under restrictive conditions with selection for Km^r, Spc^r, and Sm^r (and screening for Cm^s in strain HD30). Strain MN7 harboring plasmid pB3Ω was capable of growth at 42°C as described in Materials and Methods. When introduced into strain HD30, plasmid pB3Ω was able to replace plasmid pHD102 as the covering plasmid. Southern blot analysis of strain HD30/pB3Ω confirmed that the chromosomal copy of the $pgsA_{Ec}$ gene was still disrupted and neither plasmid pHD102 nor an intact copy of the $pgsA_{Ec}$ gene was present (data not shown).

PgsA activity was assayed in three *E. coli* strains (DH5α [$pgsA^+$] and the two *pgsA* mutant strains MN7 and HD30) either with or without plasmid pB3Ω (Table 2). In all cases the introduction of plasmid pB3Ω increased PgsA enzymatic activity above the vector and non-plasmid-containing control levels. Particularly significant was the high heat stable activity in strain MN7/pB3Ω cell extracts and the presence of activity in strain HD30/pB3Ω lacking plasmid pHD102. The addition of IPTG to induce the *lac* promoter on plasmid pB3Ω did not result in additional enzyme activity over that found in uninduced cells.

Phospholipid composition of *R. sphaeroides* and *E. coli* strains. Total phospholipids were isolated and analyzed by thin-layer chromatography from ^{32}P-labeled cultures of *R. sphaeroides* either with or without plasmid pB1, of *E. coli*

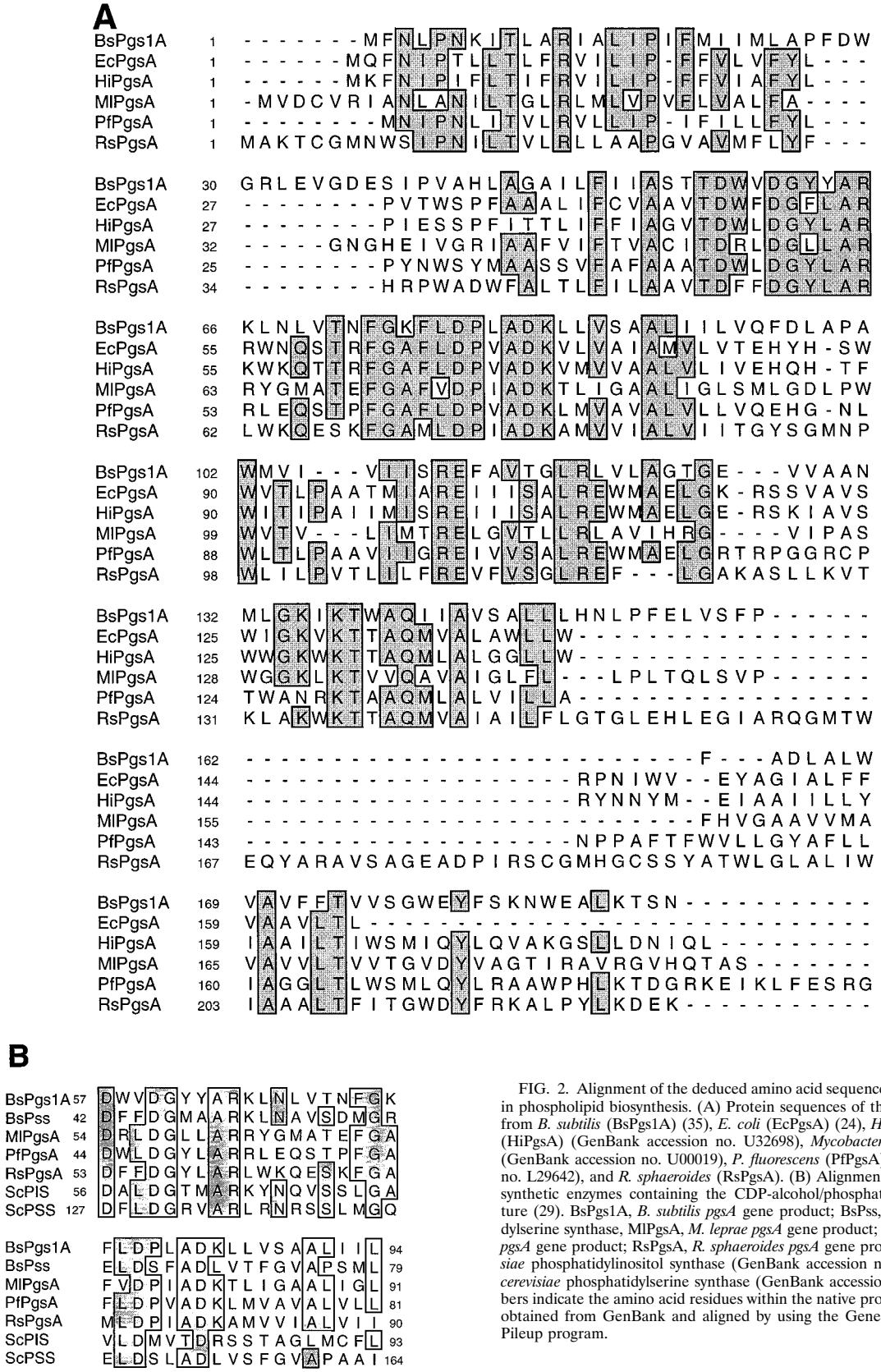

FIG. 2. Alignment of the deduced amino acid sequences of proteins involved in phospholipid biosynthesis. (A) Protein sequences of the *pgsA* gene products from *B. subtilis* (BsPgs1A) (35), *E. coli* (EcPgsA) (24), *Haemophilus influenzae* (HiPgsA) (GenBank accession no. U32698), *Mycobacterium leprae* (MlPgsA) (GenBank accession no. U00019), *P. fluorescens* (PfPgsA) (GenBank accession no. L29642), and *R. sphaeroides* (RsPgsA). (B) Alignment of phospholipid biosynthetic enzymes containing the CDP-alcohol/phosphatidyltransferase signature (29). BsPgs1A, *B. subtilis pgsA* gene product; BsPss, *B. subtilis* phosphatidylserine synthase, MlPgsA, *M. leprae pgsA* gene product; PfPgsA, *P. fluorescens pgsA* gene product; RsPgsA, *R. sphaeroides pgsA* gene product; ScPIS: *S. cerevisiae* phosphatidylinositol synthase (GenBank accession no. J02697); ScPSS: *S. cerevisiae* phosphatidylserine synthase (GenBank accession no. U18778). Numbers indicate the amino acid residues within the native proteins. Sequences were obtained from GenBank and aligned by using the Genetics Computer Group Pileup program.

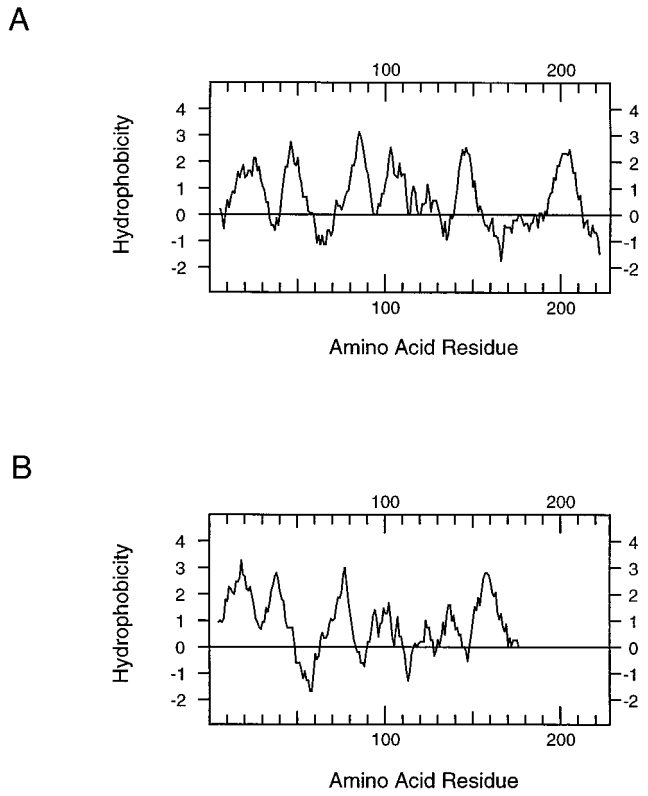

FIG. 3. Hydropathy profiles of the $pgsA_{Rs}$ (A) and $pgsA_{Ec}$ (B) gene products. The base line represents the average hydropathy value for several soluble proteins determined as described by Kyte and Doolittle (38) (hydrophobic is positive); data are averages.

TABLE 2. PgsA activity

Strain/plasmid	Sp act[a]
R. sphaeroides[b]	
2.4.1/pRK415	1.8
2.4.1/pRK1	12.2
2.4.1/pBBR1MCS-2	1.8
2.4.1/pB1	15.6
2.4.1/pB2	12.8
2.4.1/pB3	8.7
E. coli[c]	
DH5α/pBBR1MCS-2	4.6
DH5α/pB3Ω	8.1
MN7	0.18
MN7/pB3Ω	6.4
HD30/pHD102	6.9
HD30/pB3Ω	8.5

[a] Strains were assayed at 37°C, as described in Materials and Methods, after a 20-min incubation of the cell lysates at 50°C. Specific activities (units per milligram of protein) are the averages of duplicate measurements of three separate experiments. The results of each set of experiments were within ±2%.

[b] Strains were grown aerobically at 30°C as described in Materials and Methods.

[c] Strains MN7 and HD30/pHD102 were grown at 30°C; all others, including MN7/pB3Ω and HD30/pB3Ω, were grown at 37°C.

DH5α and MN7 either with or without plasmid pB3Ω, and of HD30/pHD102 or HD30/pB3Ω. *R. sphaeroides*/pB1 was somewhat higher in acidic phospholipids than *R. sphaeroides* without the plasmid, but this difference was not in proportion to the sevenfold increase in PgsA enzymatic activity (Table 3); a similar small increase in acidic phospholipids content has been observed when $PsgA_{Ec}$ is overexpressed in *E. coli* (47). Plasmid pB3Ω had little effect on the phospholipid composition of *E. coli* DH5α, returned strain MN7 to a more typical wild-type level of acidic phospholipids, and was able to substitute for plasmid pHD102 in maintaining the acidic phospholipid content of strain HD30.

Addition of arbutin to the growth media of *E. coli* has been shown to increase the flux of intermediates through the phosphatidylglycerol pool about sevenfold without having a dramatic effect on the steady-state level of phosphatidylglycerol (31). This change in flux has been attributed to the increase in turnover of phosphatidylglycerol due to arbutin serving as an analog of the natural acceptor of glycerophosphate transferred from the donor (phosphatidylglycerol) in membrane-derived oligosaccharide biosynthesis. The effects of arbutin on strain HD30 expressing $PgsA_{Ec}$ and on the same strain expressing $PgsA_{Rs}$ were the same, i.e., a reduction in the cardiolipin levels, presumably due to diversion of phosphatidylglycerol to modification of arbutin, with little change in phosphatidylglycerol levels. Therefore, the $PgsA_{Rs}$ activity, like the $PgsA_{Ec}$ activity, can be adjusted to accommodate large changes in flux through the phosphatidylglycerol pool.

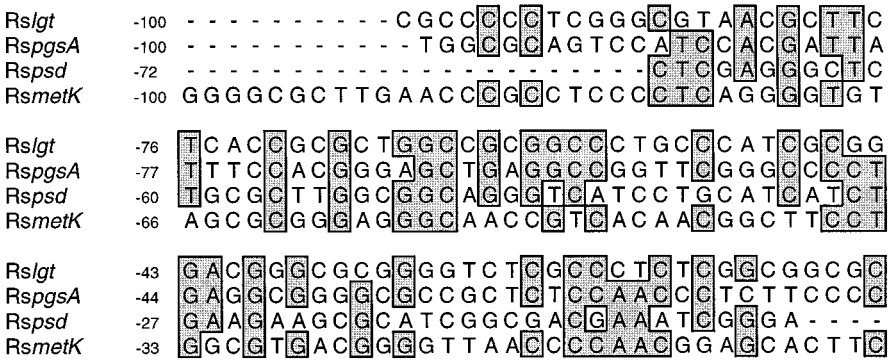

FIG. 4. Alignment of upstream DNA sequences from four *R. sphaeroides* genes. The DNA sequence 5′ of the ATG start codon of each gene was aligned by using the Genetics Computer Group Pileup program. Abbreviations: Rs*lgt*, *R. sphaeroides* homolog to the *E. coli lgt* gene encoding phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (17, 22); Rs*pgsA*, *R. sphaeroides* gene encoding PgsA; Rs*psd*, possible *R. sphaeroides* homolog to the *E. coli* phosphatidylserine decarboxylase gene (17, 39); and Rs*metK*, possible *R. sphaeroides* homolog to the yeast *metK* gene, encoding *S*-adenosylmethionine synthetase (17, 59).

TABLE 3. Phospholipid composition[a]

Strain/plasmid[a]	Mol% of[b]:					
	PG	PC	PE	CL	Other phospholipids[c]	Acidic phospholipids[d]
2.4.1	33	16	40	5	5	38
2.4.1/pB1	41	14	41	4	3	45
HD30/pHD102	15		68	8	1	23
With arbutin	15		84	0.6	0	16
HD30/pB3Ω	15		71	12	2	27
With arbutin	18		78	2	0	20
MN7	11		73	10	7	21
MN7/pB3Ω	20		68	8	5	28
DH5α	8		70	19	2	27
DH5α/pB3Ω	10		72	14	2	24

[a] Strains were grown aerobically and phospholipid composition was determined as described in Materials and Methods. *R. sphaeroides* strains, MN7, and HD30/pHD102 were grown at 30°C; all other *E. coli* strains were grown at 37°C.
[b] Abbreviations: PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin. Values are reported assuming one phosphate per mole of "other" phosphate-containing material. Experiments were performed at least twice, and the results of each set of experiments were within 5% of each other.
[c] Includes phosphatidylserine, phosphatidic acid, and CDP-diacylglycerol.
[d] Phosphatidylglycerol plus cardiolipin.

DISCUSSION

Utilizing a plasmid potentially containing a portion of the $pgsA_{Rs}$ homolog of the $pgsA_{Ec}$ gene, we were able to clone the entire gene from an *R. sphaeroides* cosmid bank. The DNA insert in cosmid pLAB14 maps to *Ase*I fragment B on chromosome I of *R. sphaeroides* (41). DNA sequence analysis revealed the presence of one complete ORF with a high degree of homology between $PgsA_{Rs}$ and the products of the *pgsA* genes of not only *E. coli* (24, 61) but also a number of other bacteria as shown in Fig. 2A; however, except for *E. coli*, no functional analysis has been reported for these other gene products. As in *E. coli*, it appears that although the *uvrC* and *pgsA* genes are arranged in tandem, the two genes are not within a common operon and each has its own promoter, since the *pgsA* genes are functional without an intact *uvrC* ORF. In *E. coli* the *pgsA* promoter is buried in the 3′-terminal region of the *uvrC* gene (24) and 56 bp separate the termination codon of *uvrC* and the start codon of the *pgsA* gene. In *R. sphaeroides* there are either 81 or 96 bp separating *uvrC* from *pgsA*.

No promoter sequences could be identified when the upstream sequence was compared with the classic *E. coli* σ^{70} (30) or with the promoter regions found upstream of the rrn_{Rs} operons (19). However, when upstream sequences of three other *R. sphaeroides* ORFs were aligned with that of $pgsA_{Rs}$, some regions of homology were identified (Fig. 4). All four genes in Fig. 4 (*lgt*, *pgsA*, *psd*, and *metK*) are believed to be involved in phospholipid metabolism (17) and are probably essential for normal cell function as has been found in *E. coli* (16, 59). Therefore, the regions of homology found upstream of the start of each of these genes may be regulatory signals important in either transcriptional or translational control for "housekeeping genes." Experiments are currently under way to identify the promoter and any regulatory regions upstream of the $pgsA_{Rs}$ gene.

When *R. sphaeroides* carrying plasmid pB1 ($pgsA_{Rs}$ gene) was assayed for enzyme activity, a sevenfold increase in activity over the wild-type level was seen. This suggests strongly that the $pgsA_{Rs}$ promoter is present either within the 3′ end of the *uvrC* ORF or in the spacer region between the two genes. In the hope that expression of the $pgsA_{Rs}$ gene could be increased even more, the $rrnB_{Rs}$ promoter was inserted 12 bp upstream of the first start codon of the $pgsA_{Rs}$ gene; this promoter, when fused to other genes, can increase activity up to 20-fold in *R. sphaeroides* (17, 19). However, no increase above the activity found with plasmid pB1 was seen. Similarly, when plasmid pRK1 was introduced into *R. sphaeroides*, it too produced the same level of enzyme activity as plasmid pB1. Replacement of the native promoter with the lac_{Ec} promoter reduced the level of enzymatic activity expressed, which is consistent with the generally poor ability of *R. sphaeroides* RNA polymerase to read *E. coli* promoters (19). The above-mentioned plasmids all differ in the sequences upstream of the putative promoters driving the expression of the $pgsA_{Rs}$ gene. However, only replacing the putative $pgsA_{Rs}$ promoter with the lac_{Ec} promoter resulted in significantly lower expressed activity. This result is consistent with the regions immediately upstream of the gene acting as promoters rather than regions further upstream. These results also suggest an upper limit for expression of functional enzyme of about seven times the wild-type levels. Clearly, further work is needed to sort out whether this limit on the level of activity is at the transcriptional level, at the translational level, or due to posttranslational events.

When plasmid pRK1, pB1, or pB2 was introduced into *E. coli pgsA* mutant strains, there was no complementation of phenotype or detectable enzymatic activity dependent on these plasmids, again consistent with the poor recognition of *R. sphaeroides* promoters by *E. coli* RNA polymerase (19). However, when plasmid pB3Ω, which has the $pgsA_{Rs}$ gene under the control of the lac_{Ec} promoter, was introduced into *E. coli pgsA* mutant strains MN7 and HD30 under restrictive conditions, the plasmid was able to rescue the conditional lethal phenotypes of both strains. Since the latter strain carries a null allele of the essential $pgsA_{Ec}$ gene, the $pgsA_{Rs}$ gene product can substitute for the *E. coli* enzyme, which was verified by levels of PgsA activity in the range found in wild-type *E. coli.*

The above activity and complementation results demonstrate the feasibility of cloning genes involved in phospholipid biosynthesis in *R. sphaeroides* by complementation of conditional lethal mutations in the homologous genes of *E. coli*. Success in using this approach to isolate such clones will depend on use of expression libraries driven by an *E. coli* promoter such as *lac*, which we are currently constructing; failure to obtain complementation of *E. coli* mutants by the gene under the control of an *R. sphaeroides* promotor emphasizes the need to develop *R. sphaeroides* DNA libraries which can be expressed in *E. coli* in order to utilize complementation as a means of gene isolation. The added advantage of the lac_{Ec} promoter is that it is also functional in *R. sphaeroides*, which will allow the direct transfer of the cloned gene in the appropriate vector into *R. sphaeroides* for verification of function in its native organism. The availability of this approach will be very useful for isolating other genes involved in phospholipid biosynthesis in *R. sphaeroides*, as well as other diverse bacteria.

Several other examples of complementation of *E. coli* genes with mutations in phospholipid metabolism by genes from other organisms exist. Both the *Bacillus subtilis pss* (48) and *B. subtilis pgsA* (35) genes complement mutant genes of *E. coli*, but in both cases the natural promoters were functional, which may be the general case for *B. subtilis* promoters; in the latter case complementation was of a defect in protein translocation and not phospholipid metabolism. The *Saccharomyces cerevisiae PSD1* gene will complement a mutant psd_{Ec} gene, but in this case an *E. coli* promoter is required for expression (9). These examples demonstrate the utility of the nearly complete collection of *E. coli* mutants with altered phospholipid metab-

olism for isolating genes important to phospholipid metabolism from a spectrum of organisms.

Expression of $PgsA_{Rs}$ activity in $pgsA_{Ec}$ mutants at levels 1.5- to 2-fold higher than wild-type levels was sufficient to maintain normal phosphatidylglycerol and cardiolipin levels (even in the presence of arbutin), which are reduced at least 10-fold under the restrictive growth conditions for these mutants (27, 54). This result suggests either that there is no regulation of the committed step to anionic phospholipid synthesis in *E. coli* or that the *R. sphaeroides* enzyme responds to the same regulatory factors as does the *E. coli* enzyme. The latter seems to be the case and most likely occurs through regulation of the activity of PgsA. In normal *E. coli* cells, PgsA activity appears to be in excess since overproduction of the enzyme up to 20-fold only slightly elevates the steady-state level of anionic phospholipids (47), and down regulation of the expression of the $pgsA_{Ec}$ gene results in a decrease in anionic phospholipid content only after the PgsA level drops below about 15% of wild-type levels (27). Finally, the metabolic flux through the phosphatidylglycerol pool of wild-type *E. coli* can vary over a sevenfold range (31) by the addition of arbutin to the growth medium without changing the steady-state level of either $PgsA_{Ec}$ or phosphatidylglycerol. Similarly, the addition of arbutin to *E. coli* cells dependent on $PgsA_{Rs}$ for growth did not reduce their phosphatidylglycerol content, supporting the conclusion that there is excess potential activity of PgsA in these cells, which can respond to the normal signals regulating phospholipid metabolism in *E. coli*. These results are in marked contrast to the heterologous complementation of a null allele of the pss_{Ec} (encoding phosphatidylserine synthase, the committed step to phosphatidylethanolamine biosynthesis) by the *pss* gene from *B. subtilis* (48). The *B. subtilis* gene product appears not to be regulated in *E. coli*, since there was a marked increase in the steady-state level of phosphatidylethanolamine, which is not surprising given the lack of any similarity between the *B. subtilis* and *E. coli* phosphatidylserine synthases (15, 48).

In conclusion, we have isolated and cloned the *R. sphaeroides pgsA* gene and expressed the gene in *R. sphaeroides*. Functional expression in *E. coli* occurred only when an *E. coli* promoter was used, which demonstrates the potential for using *E. coli* mutants for cloning the homologous genes from *R. sphaeroides*. We are currently also constructing an *R. sphaeroides pgsA* null mutant and designing experiments in which we can further manipulate the synthesis of $PgsA_{Rs}$ to study the effects on intracytoplasmic membrane formation when the amount of phosphatidylglycerol is greatly reduced in *R. sphaeroides.*

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