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Pseudomonas aeruginosa Synthesizes Phosphatidylcholine by Use of the Phosphatidylcholine Synthase Pathway

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Phosphatidylcholine (PC) is a ubiquitous membrane lipid in eukaryotes but has been found in only a limited number of prokaryotes. Both eukaryotes and prokaryotes synthesize PC by methylating phosphatidylethanolamine (PE) by use of a phospholipid methyltransferase (Pmt). Eukaryotes can synthesize PC by the activation of choline to form choline phosphate and then CDP-choline. The CDP-choline then condenses with diacylglycerol (DAG) to form PC. In contrast, prokaryotes condense choline directly with CDP-DAG by use of the enzyme PC synthase (Pcs). PmtA was the first enzyme identified in prokaryotes that catalyzes the synthesis of PC, and Pcs in *Sinorhizobium meliloti* was characterized. The completed release of the *Pseudomonas aeruginosa* PAO1 genomic sequence contains on open reading frame predicted to encode a protein that is highly homologous (35% identity, 54% similarity) to PmtA from *Rhodobacter sphaeroides*. Moreover, the *P. aeruginosa* PAO1 genome encodes a protein with significant homology (39% amino acid identity) to Pcs of *S. meliloti*. Both the *pcs* and *pmtA* homologues were cloned from PAO1, and homologous sequences were found in almost all of the *P. aeruginosa* strains examined. Although the pathway for synthesizing PC by use of Pcs is functional in *P. aeruginosa*, it does not appear that this organism uses the PmtA pathway for PC synthesis. We demonstrate that the PC synthesized by *P. aeruginosa* PAO1 localized to both the inner and outer membranes, where it is readily accessible to its periplasmic, PC-specific phospholipase D.

Phosphatidylcholine (PC) is the major membrane-forming phospholipid in most eukaryotes; however, it is found in only a small but increasing number of prokaryotes where phosphatidylethanolamine (PE) serves as the major membrane-forming phospholipid. As shown in Fig. 1, both eukaryotes and prokaryotes synthesize PC by use of the methylation pathway in which PE is sequentially methylated three times by use of the methyl donor *S*-adenosylmethionine (SAM) and the enzyme phospholipid *N*-methyltransferase (Pmt). In addition to this methylation pathway in eukaryotes, there is a pathway by which choline is converted to choline-phosphate and then to CDP-choline, which subsequently condenses with diacylglycerol (DAG) to form PC (Kennedy pathway) (17). In prokaryotes, the presence of a methyltransferase was detected in *Agrobacterium tumefaciens* (16), and thereafter Arondel et al. isolated a 22.9-kDa soluble protein (PmtA) from *Rhodobacter sphaeroides*, which when expressed in *Escherichia coli* (an organism deficient of PC and methylated derivatives of PE), resulted in the accumulation of PC (2). Consequently, only the methylation pathway was thought to exist in prokaryotes (28) until de Rudder et al. demonstrated a novel pathway (Fig. 1) in the soil bacterium *Sinorhizobium meliloti* that generates PC by use of the enzyme PC synthase (Pcs) to condense choline directly with CDP-DAG (7, 8).

The impact of lipid composition on bacterial growth is not sufficiently understood, although it is thought to contribute to the interaction between the bacteria collectively called rhizobia (e.g., *Rhizobium*, *Sinorhizobium*, and *Bradyrhizobium* spp.) and the roots or stems of leguminous host plants, leading to a symbiotic development. Individual lipid composition often defines the stability and integrity of membrane lipids (25), and environmental conditions such as oxygen tension can affect lipid composition. For example, Tang and Hollingsworth reported that lowering the oxygen tension in free-living *Bradyrhizobium japonicum* results in an increase in synthesis of PE and phosphatidylglycerol and a decrease in PC synthesis (34). Minder et al. (20) further showed that unlike the decreased growth observed in a sinorhizobial strain deficient in PC (6), the growth of *B. japonicum* mutants with decreased PC synthesis was largely unaffected. However, maintaining a wild-type amount of PC in the membrane is required for an efficient symbiotic interaction of *B. japonicum* with its soybean host plant. Furthermore, it is expected that sinorhizobial PC will be required for the formation of *B. japonicum*'s successful symbiosis with its plant host (6). It has been suggested that only highly specialized groups of bacteria, mainly photosynthetic bacteria containing extensive internal membrane structures or those living in association with eukaryotes, such as *B. japonicum* and *S. meliloti*, contain PC as a membrane lipid (11). However, to date, PC-containing bacteria have been found in distantly related groups, like gram-positive bacteria (e.g., *Cellulomonas* and *Hongia* spp.), bacteroides-flavobacterium group bacteria (e.g., *Cyclobacterium* and *Flexibacter* spp.), and spirochetes (e.g., *Borrelia* and *Treponema* spp.), indicating that PC is found in more bacteria than was originally thought (18).

Pseudomonas aeruginosa, a gram-negative opportunistic pathogen, is especially problematic for those predisposed to

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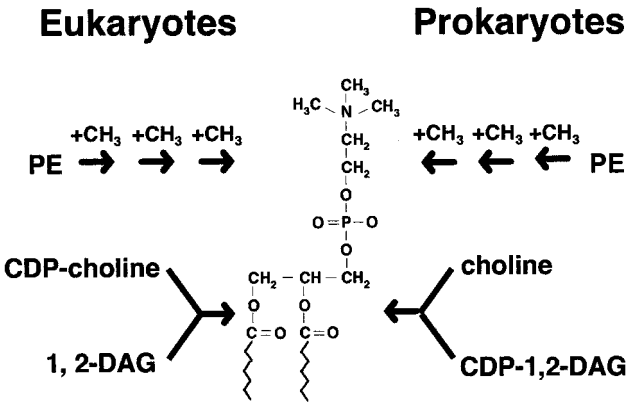

FIG. 1. Biosynthetic pathways for PC in eukaryotes and prokaryotes. Eukaryotes and prokaryotes can generate PC by methylating ($+CH_3$) PE by use of Pmt. In addition, eukaryotes synthesize PC by condensing CDP-choline with DAG, while prokaryotes condense choline with CDP-DAG by use of Pcs.

respiratory infections such as those with cystic fibrosis (CF). In addition, *P. aeruginosa* can be isolated from moist soils and, while it is pathogenic to some plants (26, 27), in some cases it provides plants with protection (5). There is an open reading frame (ORF) (PA0798) in the completed release of the *P. aeruginosa* PAO1 genomic sequence (www.pseudomonas.com) (33) predicted to encode a protein that is highly homologous to PmtA from *R. sphaeroides* and an ORF (PA3857) predicted to encode a protein with significant homology to Pcs of *S. meliloti*. In this report, we cloned the *pmtA* and *pcs* genes from *P. aeruginosa* PAO1 and characterized mutants that are PC deficient.

MATERIALS AND METHODS

Bacterial strains and media. The strains and plasmids used in this study are described in Table 1. *P. aeruginosa* PAO1 is the prototypic strain and has been previously described (14). Brain heart infusion (BHI) and Luria-Bertani (LB) media (32) were used for strain maintenance. HEPES minimal medium [0.5 mM $MgSO_4$, 0.1 M HEPES, 7 mM $(NH_4)_2SO_4$, 20 mM potassium succinate, and 0.02 mM K_2HPO_4 (pH 7)] was autoclaved, and a solution containing 0.1% trace ions (139.1 mM $ZnCl_2$, 1.62 mM $MnCl_2$, 1.78 mM $FeCl_2$, 2.45 mM $CaCl_2$, 4.69 mM H_3BO_4, 0.95 mM CsCl) was added to a final concentration of 0.1% (vol/vol). In some experiments, HEPES minimal medium was supplemented with 0.2% choline. Finally, yeast extract-tryptone (2×YT; per liter: 10 g of yeast extract, 16 g of tryptone, 5 g of NaCl [pH 7]) was used when strains were cultured for PC. When appropriate, medium was supplemented with the following antibiotics at the indicated concentrations: for *E. coli*, ampicillin at 100 μg/ml, gentamicin at 15 μg/ml, kanamycin at 100 μg/ml, streptomycin at 100 μg/ml, and tetracycline at 15 μg/ml; for *P. aeruginosa*, carbenicillin at 750 μg/ml, gentamicin at 75 μg/ml, streptomycin at 750 μg/ml, and tetracycline at 150 μg/ml.

DNA manipulations and analysis. Plasmid and chromosomal DNAs were isolated by standard procedures (29). The *pmtA* and *pcs* genes from *P. aeruginosa* PAO1 were cloned as 2.1-kb *Bcl*I and 5.1-kb *Kpn*I fragments, respectively, into pBluescript SK(+) (Stratagene). *Taq* polymerase and an 18- or 23-mer primer (Gibco BRL) (Table 1) were used for PCR with a GeneMate thermal cycler. DNA sequence analysis was performed using the dideoxy chain-termination method (30) with Sequenase (United States Biochemicals).

Analysis of *P. aeruginosa* strains for *pmtA* and *pcs* sequences. PCR was performed using the primers shown in Table 1 and standard methods (29). Amplification of *pmtA* and *pcs* homologues with these internal primers results in products of 592 and 380 bp, respectively. The products were analyzed on agarose gels. To verify the quality of the DNA from each strain, a conserved methionyl tRNA synthase gene was amplified, resulting in a 1,039-bp fragment.

In vivo labeling and analysis of lipids of *P. aeruginosa*. Strains were cultured in media containing [1-^{14}C]acetate (2 μCi/ml of culture, 37.0 MBq/mCi; New England Nuclear) at 32°C for 12 to 15 h with shaking. Lipids were extracted by use of the Bligh and Dyer method (3). Briefly, cells were collected, washed with H_2O, and extracted with methanol-chloroform-water (2:2:1, vol/vol/vol). The bottom organic phase was dried, dissolved in chloroform, and spotted onto Silica Gel 60A plates (Whatman). For one-dimensional thin-layer chromatography (1D-TLC), the lipids were separated with chloroform-methanol-acetic acid (13:3:1, vol/vol/vol). For two-dimensional TLC (2D-TLC), the lipids were separated in the first phase with chloroform-methanol-water (14:6:1, vol/vol/vol), followed by separation in the second phase with chloroform-methanol-acetic acid (13:5:1, vol/vol/vol). The radiolabeled lipids were visualized using a Bio-Rad personal FX phosphorimager. Image analysis was done using Quantity One software (version 4.0.3) from Bio-Rad.

Mass spectrometry. Total lipids were labeled and extracted as described above. Lipids were suspended in 1 ml of methanol. Samples (2 μl) were injected onto a Prodigy 5μ octadecyl silane (3) 100-Å-resolution column (1.00 by 250 mm; Phenomenex, Torrance, Calif.). The high-pressure liquid chromatograph was operated at a flow rate of 50 μl/min, with solvent A consisting of methanol-acetonitrile-water (60:20:20, vol/vol/vol) containing 1 mM ammonium acetate and solvent B consisting of 1 mM methanolic ammonium acetate. Samples eluted from the column along a gradient from 50 to 99% solvent B in 25 min, after which the flow was held isocratically at 99% solvent B until 35 min. All effluent was directed to the mass spectrometer. The Finnigan (San Jose, Calif.) model LCQ ion trap mass spectrometer was used for all analyses. Spectra were acquired by three sequential scan modes. The first mode analyzed positive ions ranging from *m/z* 500 to 1,000. Next, the most-intense ions from the initial mode were analyzed at high resolution to accurately determine the mass-to-charge ratio of the molecular ion species (±0.01 atomic mass unit). Finally, collision-induced decomposition analysis (CID) was performed on the $[M + H]^+$ ions identified by the previous scan. CID was performed at an activation amplitude of 1.5 V (peak-to-peak resonance excitation RF voltage) for 30 ms to facilitate product ion analysis. The electrospray ionization source capillary was set at 200°C. MS^n experiments were carried out with an isolation width of ±1.5 Da. The *q* value was set at 0.21 in order to ascertain the presence or absence of the diagnostic-fragment ion occurring at *m/z* 184, which indicates the presence of glycerophosphocholine (GPCho).

The lipids from a 1-ml culture of PAO1 grown in 2×YT were dissolved in methanol, and 200 μl was removed and dried under vacuum centrifugation. Dried lipids were suspended in 100 μl of hexane-isopropanol-water (3:4:0.7, vol/vol/vol). From this reconstituted sample, 50 μl was injected onto an Ultremex 5 Silica (4.6- by 250-mm) column (Phenomenex). The high-pressure liquid chromatograph was operated at a flow rate of 1,000 μl/min, with solvent A consisting of hexane-isopropanol (3:4, vol/vol). Solvent B consisted of hexane-isopropanol-water (3:4:0.7, vol/vol/vol) containing 1 mM ammonium acetate. The sample was injected onto the column, which was equilibrated at 47% solvent B, and this composition was held for 6 min. We obtained a linear gradient from 47% solvent B to 100% solvent B over the next 20 min. The column was then held at 100% solvent B for 20 min. The effluent from the column was split, with 150 μl entering the mass spectrometer and the remaining sample collected in 1-min fractions.

A PE Sciex (Toronto, Canada) API 3000 mass spectrometer was utilized for the following analysis. Spectra were acquired in precursor ion mode by scanning the positive *m/z* range from 600 to 850 for precursors of the diagnostic-fragment ion occurring at *m/z* 184.2, which indicates the presence of GPCho. Instrument parameters were optimized for CID in the mass spectrometry-mass spectrometry mode. The fractions at 31, 32, 33, and 34 min contained GPCho as indicated by the presence of an ion at *m/z* 184.2 in the third quadrupole. Fraction 32 was analyzed in negative-ion mode to verify the demethylation of the GPCho-containing lipids, visualized as the $[M-15]^-$ anions. Collisional activation of the $[M-15]^-$ anions was undertaken in product ion mode to determine the fatty acid products present in each GPCho molecular species. Flow injection analysis of 10 μl from fraction 32 was performed for each $[M-15]^-$ anion.

Disruption of the *pmtA* and *pcs* genes. The *P. aeruginosa* PAO1 Δ*pmtA*, Δ*pcs*, and Δ*pmtA* Δ*pcs* strains were constructed as follows. *P. aeruginosa* PAO1 Δ*pmtA* was generated by replacing a 351-bp *Nco*I-*Nar*I fragment from the *pmtA* coding sequence with a gentamicin resistance (Gm^r) cassette or a tetracycline resistance cassette. The *P. aeruginosa* PAO1 Δ*pcs* strain was generated by replacing a 360-bp *Hin*cII fragment from the *pcs* coding sequence with a Gm^r cassette. The antibiotic cassettes flanked by *pmtA* or *pcs* sequences were then cloned into pEX100T. A triparental mating using *E. coli* HB101/pRK2013 as the helper strain (10, 31), *E. coli* SM10/pEXΔ*pmtA* or pEXΔ*pcs*, and *P. aeruginosa* PAO1 was performed, and transconjugants were isolated. *P. aeruginosa* PAO1 Δ*pmtA* Δ*pcs* was generated by replacing a 360-bp *Hin*cII fragment from the *pcs* coding

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristics[a]	Reference or source
Strains[a]		
P. aeruginosa		
PAO1 (ATCC 15692)	Prototroph, wound isolate	14
PAO1 Δ*pmtA*	PAO1 with a Gm^r cassette replacing a region of a *pmtA* homologue	This study
PAO1 Δ*pcs*	PAO1 with a Gm^r cassette replacing a region of a *pcs* homologue	This study
PAO1 Δ*pmtA* Δ*pcs*	PAO1 with a Tet^r cassette replacing a region of a *pmtA* homologue and a Gm^r cassette replacing a region of a *pcs* homologue	This study
PAO1 Δ*prpL*	PAO1 with a Gm^r cassette replacing a region of *prpL* (PA4175)	38
PA103	Prototroph	19
PAK	Prototroph	22
PA14	Human clinical isolate	26
Ps388	Prototroph, hypotoxigenic	36
WR5	Prototroph, exotoxin A deficient	36
M2	Mouse strain	32
CF2-1, AK957	Clinical isolate	Lab collection
CPs297, CH12	Isolates obtained from the Children's Hospital, Denver, Colo.	Lab collection
B34	Blood isolates from Nagasaki University Hospital, Nagasaki, Japan	13
ccu-4	Environmental strain from Horry and Georgetown Counties in South Carolina	9
E. coli		
DH5α	*hsdR recA lacZYA* ϕ80 *lacZ*ΔM15	Gibco BRL
HB101	*hsdS recA proA lacY*	Gibco BRL
SM10	Km^r, mobilizer strain	31
Plasmids		
pBluescript SK(+)	Amp^r *lacZ, E. coli* cloning vector	Stratagene
pRK2013	Km^r, conjugation helper plasmid	10
pVLT31	Broad-host-range expression vector, Tet^r	4
pVLT31-*pmtA*	pVLT31 containing the *pmtA* homologue	This study
pVLT31-*pcs*	pVLT31 containing the *pcs* homologue	This study
pVLT35	Broad-host-range expression vector, Sm^r	10
pVLT35-*pmtA*	pVLT35 containing the *pmtA* homologue	This study
pVLT35-*pcs*	pVLT35 containing the *pcs* homologue	This study
Primers[b]		
PJW20	CGATCCGTTTAGCTCTCAGCCCGA, flanking DNA for Met-tRNA synthase	38
PJW31	CATCTTCCTGGCCGTTCACGGTCAG, internal to DNA for Met-tRNA synthase	38
PJW111	CCAGCCGCTGTGCCAGGAGAGAG, internal to *pmtA*	This study
PJW112	GGCAGGTAGCCCTGTTCCAGCTC, internal to *pmtA*	This study
PJW166	CATGAGCCAGGCCGAGATCGA, internal to *pcs*	This study
PJW167	GAGGTTGACCCAGGGGTGCAG, internal to *pcs*	This study

[a] Abbreviations: Amp^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Tet^r, tetracycline resistance; *mob*, mobilization site; *oriT*, origin of transfer (RK2).
[b] All primers were synthesized by Gibco BRL.

sequence with a Gm^r cassette in a *P. aeruginosa* Δ*pmtA* strain. A triparental mating was performed as described above using *P. aeruginosa* Δ*pmtA* as the target strain. All mutations were confirmed by PCR analysis and Southern blot hybridization.

Recovery of strains after storage in glycerol at −70°C. Bacterial strains (PAO1 and PAO1 Δ*pmtA*, Δ*pcs*, Δ*pmtA* Δ*pcs*, and Δ*prpL* mutants) were cultured in BHI for 18 h at 32°C with shaking. Viable counts of the culture were determined by plating serial dilutions on solid media without antibiotics. Glycerol stocks were made by mixing 0.85 ml of the culture with 0.15 ml of 100% glycerol and were stored at −70°C. To determine viability after freezing, the glycerol stocks were completely thawed and serial dilutions were plated as described above.

RESULTS

Identification of homologues to *pmtA* and *pcs* in *P. aeruginosa* spp. Previously, we identified a PC-hydrolyzing phospholipase D (PldA) of *P. aeruginosa* PAO1 and localized it to the periplasmic space (40). In this location it would not likely have access to eukaryotic PC. However, if *P. aeruginosa* is able, like a limited number of prokaryotes, to synthesize PC, then the periplasmic PLD would have access to an appropriate substrate. To begin to address this issue, we examined the completed release of the PAO1 genomic sequence for a homologue of PmtA, the enzyme responsible for methylating PE three successive times to generate PC in *R. sphaeroides*. The *P. aeruginosa* PAO1 genome contains an ORF (PA0798) predicted to encode an ~23.5-kDa protein that is highly homologous (35% identity, 54% similarity) to PmtA from *R. sphaeroides* (Fig. 2A). This predicted protein also contains the conserved region corresponding to the consensus motif for *S*-adenosylmethionine (SAM)-utilizing methyltransferases (12) thought to be involved in binding SAM (15). However, the N terminus of PmtA in *P. aeruginosa* is considerably more hydrophobic than the corresponding region of *R. sphaeroides* PmtA (Fig. 2C).

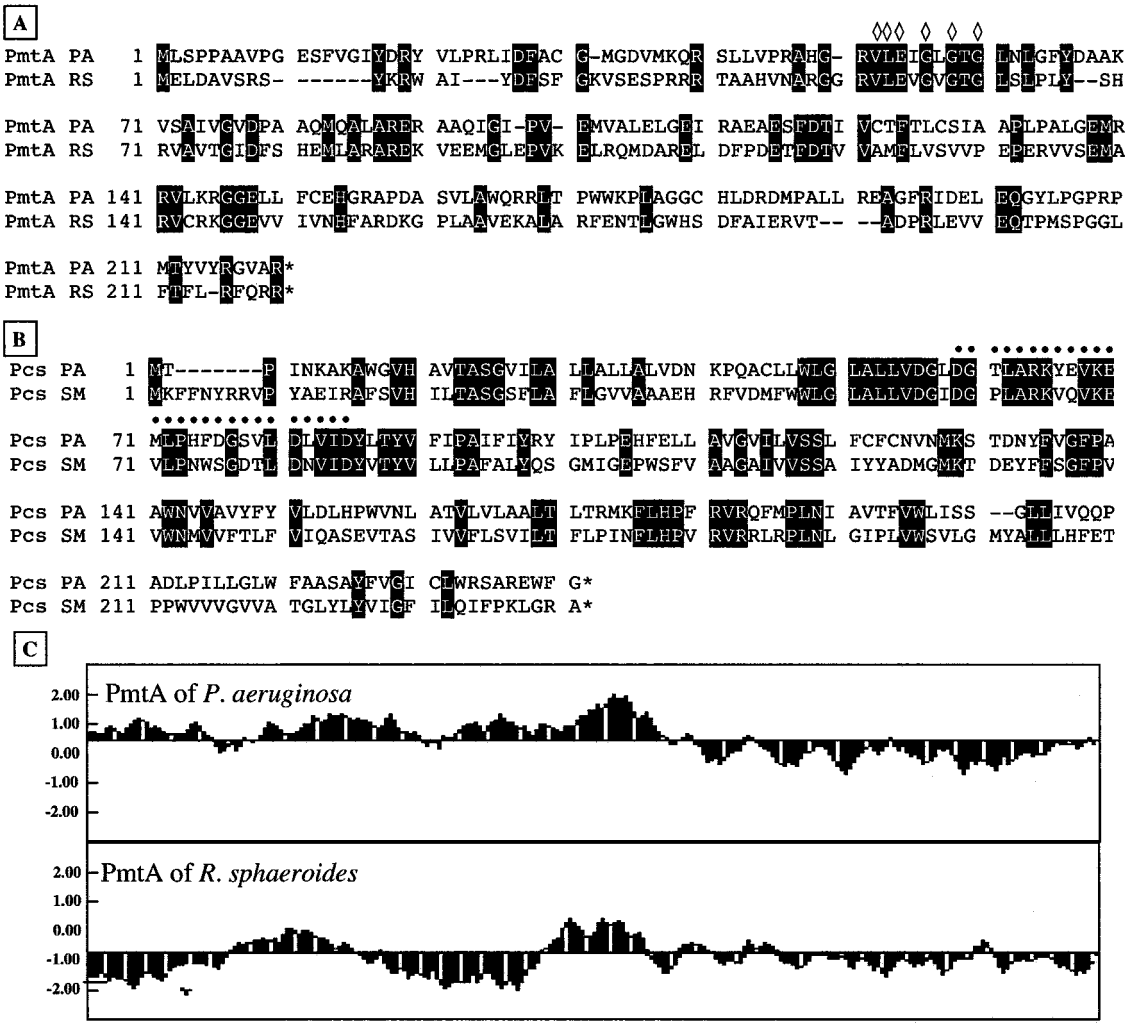

FIG. 2. Homologues of PmtA and Pcs. (A) Alignment of the PmtA homologue from *P. aeruginosa* PAO1 (PA) with the Pcs from *R. sphaeroides* (RS). (B) Alignment of the Pcs homologue from *P. aeruginosa* PAO1 with PmtA from *S. meliloti* (SM). Identical residues are boxed, and an asterisk indicates the stop codon. The diamonds in PmtA homologues indicate residues corresponding to the consensus motif for SAM-utilizing methyltransferases and thought to be involved in the binding of SAM. The black circles indicate residues comprising the motif for CDP-alcohol phosphatidyltransferases. (C) Hydropathy plots of the PmtA of *P. aeruginosa* and *R. sphaeroides*. Hydrophobic residues are depicted above the baseline, and hydrophilic residues are depicted below the baseline.

PC in prokaryotes can also be generated by the condensation of CDP-DAG and choline by the enzyme Pcs. The *P. aeruginosa* PAO1 genome encodes a protein (encoded by PA3857) with significant homology (39% amino acid identity) to Pcs of *S. meliloti* (Fig. 2B). This ~38.3-kDa predicted protein encoded by *pcs* in *P. aeruginosa* contains the motif described as being characteristic for CDP-alcohol phosphatidyltransferase ($DGX_2ARX_7GX_3DX_4D$) (40), suggesting that it may indeed play a role in PC synthesis.

To investigate the prevalence of the *pmtA* and *pcs* homologues among various clinical and environmental strains of *P. aeruginosa*, we analyzed these strains using PCR with primers specific for internal regions of these homologues (Table 1). Of the 13 strains analyzed, 12 carry sequences homologous to *pmtA* while all of the strains examined carry sequences homologous to *pcs* (data not shown). Furthermore, a region from a conserved tRNA gene was amplified in all of the strains examined, verifying the quality of the chromosomal template used for PCR (data not shown).

***P. aeruginosa* PAO1 synthesizes PC.** The identification of *pmtA* and *pcs* homologues in the PAO1 genome suggests that this organism is capable of synthesizing PC by using PmtA in the methylation pathway and by using Pcs to condense choline and CDP-DAG. Previous studies have indicated that *P. aeruginosa* does indeed contain PC (1, 39); however, the mechanisms and genes involved in PC synthesis have not been elucidated. To verify that PC is present in PAO1, cells were cultured in 2×YT in the presence of [1-^{14}C]acetate and total lipids were analyzed using 2D-TLC. As shown in Fig. 3A and B, *P. aeruginosa* PAO1 synthesizes a lipid that comigrates with L-α-dipalmitoyl-[dipalmitoyl-1-^{14}C]PC (New England Nuclear). To confirm that this comigrant is indeed PC, it was extracted from the silica of the TLC plate and subjected to digestion with the purified PC-specific hydrolyzing phospholipase C (PC-PLC)

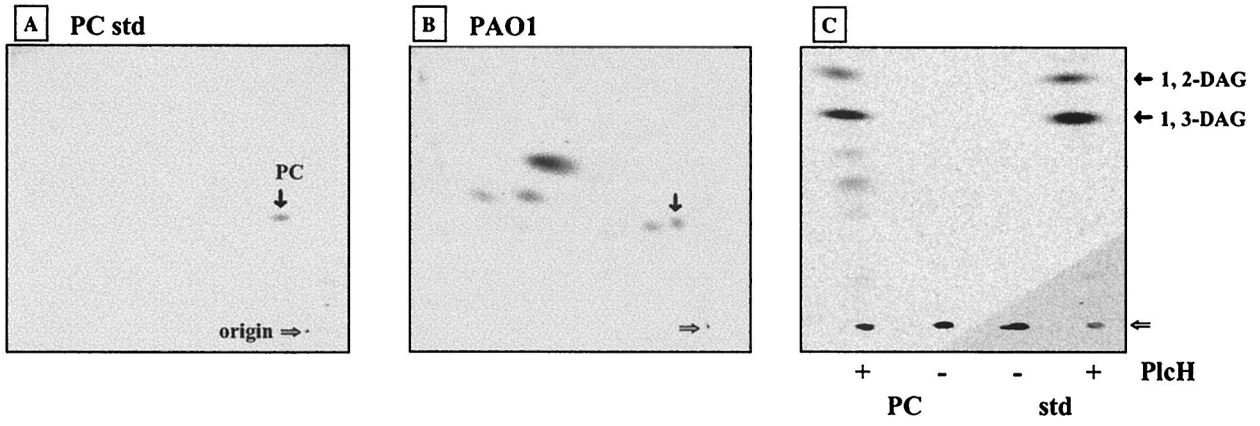

FIG. 3. 2D-TLC analysis of total lipids from *P. aeruginosa* PAO1. Migrations of commercially available L-α-dipalmitoyl-[dipalmitoyl-1-^{14}C]PC (A) and [1-^{14}C]acetate-labeled lipids (B) from *P. aeruginosa* PAO1 are shown. Cells were cultured in the presence of [1-^{14}C]acetate, and lipids were extracted and separated using 2D-TLC. (C) Digestion with PlcH of the lipid spot comigrating with PC. The labeled lipid comigrating with PC and commercially available L-α-dipalmitoyl-[dipalmitoyl-1-^{14}C]PC (std) were extracted from the TLC plate and digested with purified PC-PLC from *P. aeruginosa* PAO1. Digestion products were separated using 1D-TLC. The positions of the loading origin (open arrow), PC (filled arrows), and 1,2- and 1,3-DAG are indicated.

from *P. aeruginosa* PAO1 (M. J. Stonehouse, A. Cota-Gomez, S. K. Parker, W. E. Martin, J. A. Hankin, R. C. Murphy, W. Chen, K. B. Lim, M. Hackett, A. I. Vasil, and M. L. Vasil, submitted for publication). Digestion of the extracted lipid and the commercially available L-α-dipalmitoyl-[dipalmitoyl-1-^{14}C]PC yielded identical products (1,2- and 1,3-DAG), indicating that *P. aeruginosa* PAO1 contains PC (Fig. 3C). Furthermore, we examined the lipids from seven other *P. aeruginosa* strains for PC. While all of these strains produce PC, one of them does not carry a *pmtA* homologue (see above). However, all contain a *pcs* homologue (data not shown).

Mass spectrometry. The lipid that comigrates with L-α-dipalmitoyl-[dipalmitoyl-1-^{14}C]PC was also examined by mass spectrometry. Specific molecular $[M + H]^+$ ion species observed and analyzed with a mass spectrometer supported the idea that GPCho lipid molecular species contain ratios of total numbers of carbon atoms to double bonds in both fatty acid acyl substituents of 32:1, 32:0, 34:2, 34:1, 35:2, 35:1, and 36:2 (Table 2). CID of one major GPCho molecular species having a $[M + H]^+$ ion at *m/z* 760.6 yielded the major product ion at *m/z* 184.2, which was consistent with a GPCho polar head group (Fig. 4). The ions at *m/z* 478.4 and 522.6 suggested the elimination of neutral octadecenoic acid and the neutral hexadecanoic acid ketene from the precursor molecular ion species, respectively, as was expected for the 34:1 phospholipid molecular species 1-octadecenoyl-2-hexadecanoyl-*sn*-GPCho.

Localization of PC. To localize the PC present in *P. aeruginosa* PAO1, cells were cultured in 2×YT in the presence of [1-^{14}C]acetate. The inner and outer membranes of the cells were radiolabeled and fractionated according to published methods (21) and analyzed by TLC. PC was detected in both the inner and outer membranes of PAO1 (data not shown).

TABLE 2. Potential PC molecular species in *P. aeruginosa* PAO1

$[M + H]^+$ mass	GPCho[a]	Possible molecular species[b,c]
732.6	14:0; 18:1	1-Tetradecanoyl-2-octadecenoyl-*sn*-GPCho
	16:0; 16:1[d]	1-Hexadecenoyl-2-hexadecanoyl-*sn*-GPCho
734.6	16:0; 16:0	1,2-Dihexadecanoyl-*sn*-GPCho
758.6	16:1; 18:1	1-Octadecenoyl-2-hexadecenoyl-*sn*-GPCho
760.6	16:0; 18:1	1-Octadecenoyl-2-hexadecanoyl-*sn*-GPCho
772.6	16:1; 19:1	—[e]
	17:1; 18:1	—[e]
774.6	16:0; 19:1	—[e]
	17:0; 18:1	—[e]
786.6	18:1; 18:0	1,2-Dioctadecenoyl-*sn*-GPCho

[a] Observed positive ion, with the corresponding negative ion observed at 16 Da lower corresponding to $[M-15]^-$.

[b] Fatty acyl groups identified by collisional activation of $[M-15]^-$ to yield indicated carboxylate anions.

[c] Molecular species with glycerol esterification position assigned by relative abundance of carboxylate anion, with the most abundant derived from the *sn*-2 position.

[d] The position of the double bond in any unsaturated carboxylate anion was not determined.

[e] Minor species with insufficient material to unambiguously assign *sn*-1/*sn*-2.

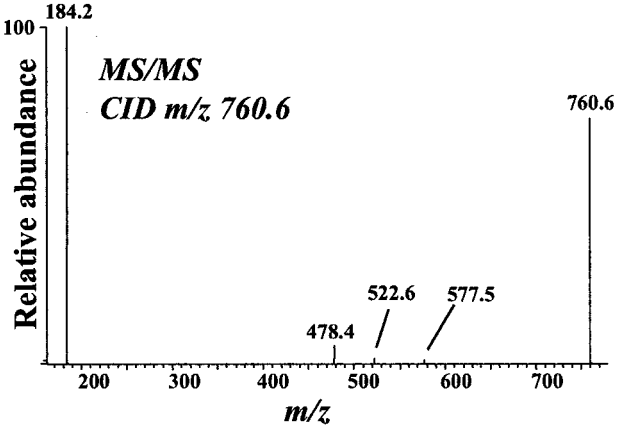

FIG. 4. Mass spectrometry. Shown are the results of analysis of the lipid that comigrates with L-α-dipalmitoyl-[dipalmitoyl-1-^{14}C]PC using mass spectrometry. CID of one major GPCho molecular species having a $[M + H]^+$ ion at *m/z* 760.6 yielded the major product ion at *m/z* 184.2, which was consistent with a GPCho polar head group.

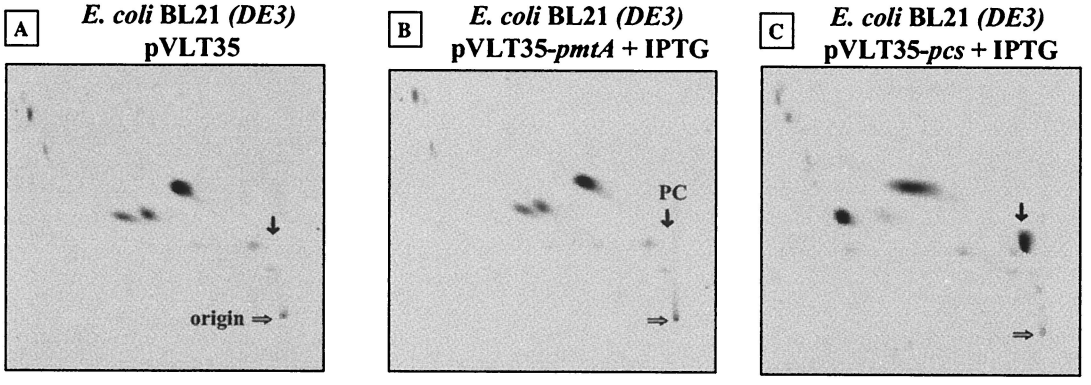

FIG. 5. Complementation of *E. coli* with *pmtA* and *pcs*. Shown are the results of analysis of the total lipids from *E. coli* BL21 (*DE3*) complemented with pVLT35 (A), pVLT35-*pmtA* (B), and pVLT35-*pcs* (C). Cells were cultured in the presence of [^{14}C]acetate, and lipids were extracted and separated using 2D-TLC. The origin (open arrows) and PC (filled arrows) are indicated.

Expression of *pmtA* and *pcs* in *E. coli*. The *pmtA* and *pcs* genes (including their predicted regulatory sequences) were cloned from *P. aeruginosa* PAO1 into pVLT35, a plasmid that replicates in both *P. aeruginosa* and *E. coli*. Using the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *Ptac* promoter of pVLT35, we expressed both the *pmtA* and *pcs* genes in *E. coli* BL21 (*DE3*), a PC-deficient organism. When the lipids from *E. coli* BL21 (*DE3*) containing pVLT35 alone or pVLT35-*pmtA* grown in 2×YT with or without IPTG were examined, PC was not detected (Fig. 5A and B and data not shown). In contrast, PC was detected when *E. coli* BL21 (*DE3*) was transformed with pVLT35-*pcs* (Fig. 5C). These results indicate that under these conditions, the production of PC in *E. coli* BL21 (*DE3*) is dependent upon expression of *pcs* but not *pmtA* and that the uninduced (without IPTG) expression of *pcs* resulted in sufficient Pcs to detect PC in lipid fractions.

Disruption of the *pmtA* and *pcs* genes in *P. aeruginosa* PAO1 and complementation. Although no PC was detected in *E. coli* transformed with pVLT35-*pmtA*, it may be that PmtA is simply not active in *E. coli* or that PmtA has a function other than methylation of PE. One indication that PmtA may be inactive results from comparing the hydropathy plots of *R. sphaeroides* PmtA and the *P. aeruginosa* PmtA homologue (Fig. 2C). Although the plots are similar, the N terminus of *P. aeruginosa* PmtA is considerably more hydrophobic than the corresponding region in *R. sphaeroides* PmtA. To address whether PmtA and Pcs are required for PC synthesis in *P. aeruginosa* PAO1, we generated *pmtA* and *pcs* mutant strains of PAO1 by removing internal portions of *pmtA* and *pcs* and replacing each with an antibiotic cassette. The lipids from these mutant strains were then grown in 2×YT and examined for PC. While PC is detected when there is a mutation in *pmtA* (Fig. 6B), a mutation in the *pcs* gene alone completely abolished the ability of *P. aeruginosa* PAO1 to synthesize PC (Fig. 6C). A mutant containing deletions in both the *pcs* and *pmtA* genes also produced no detectable PC (Fig. 6D). Furthermore, when the lipids from a Δ*pmtA* Δ*pcs* strain were analyzed using mass spectroscopy, PC was not detected (data not shown). These strains were complemented with plasmids containing the appropriate genes and then analyzed for PC production. When PAO1 Δ*pcs* was complemented with pVLT35-*pcs*, PC production was restored. Similarly, PC production in PAO1 Δ*pmtA* Δ*pcs* was restored when it was transformed with pVLT31-*pcs* but not when it was transformed with pVLT31-*pmtA* (data not shown). Taken together, these data indicate that the *pcs* gene of *P. aeruginosa* PAO1 is solely responsible for the ability of this organism to synthesize PC and that PmtA is not necessary for PC synthesis under the conditions used in this study

Pcs condenses choline and CDP-DAG to generate PC. Further evidence for the involvement of Pcs as the primary enzyme

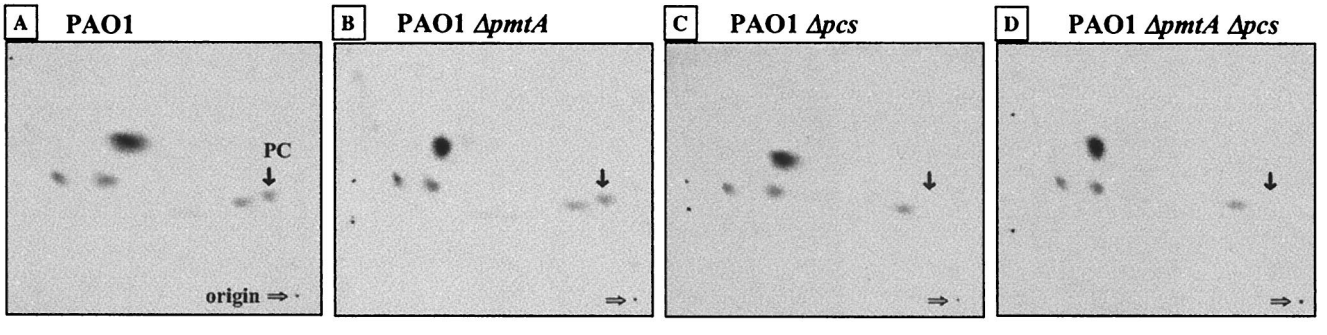

FIG. 6. Analysis of the total lipids from PAO1 and its Δ*pmtA*, Δ*pcs*, and Δ*pmtA* Δ*pcs* mutants. Cells were cultured in the presence of [1-^{14}C]acetate, and lipids from PAO1 (A) and the Δ*pmtA* (B), Δ*pcs* (C), and Δ*pmtA* Δ*pcs* (D) mutants were extracted and separated using 2D-TLC. The origin (open arrows) and PC (filled arrows) are indicated.

responsible for PC production stems from the fact that PC is produced in *P. aeruginosa* PAO1 when it is cultured in rich media (e.g., LB medium and 2×YT) or in minimal medium containing 0.2% choline (data not shown). However, PC is not detected when *P. aeruginosa* PAO1 is cultured in minimal medium without choline (data not shown), indicating that the methylation pathway is not functional even when Pcs is unable to condense choline with CDP-DAG.

Viability of *P. aeruginosa* PAO1 Δ*pmtA* and Δ*pcs* mutants. During routine handling of the strains, it was observed that the mutant strains were more difficult to recover from stocks stored in 15% glycerol at −70°C. Strains were cultured for 18 h and plated onto solid media without antibiotics. After 18 h of growth in BHI, *P. aeruginosa* PAO1 and its Δ*pmtA*, Δ*pcs*, and Δ*pmtA* Δ*pcs* mutants all had similar counts of approximately 10^8 viable CFU/ml. Following storage in 15% glycerol at −70°C, the recovery of PAO1 was 81% ± 6%. When the *pmtA* gene was deleted, recovery decreased to 63% ± 3%. The PC-deficient Δ*pcs* and Δ*pmtA* Δ*pcs* mutants experienced an even greater reduction in recovery (38% ± 2% and 16% ± 6%, respectively). A disruption in an unrelated gene (*prpL*, PA4175) containing a Gm^r cassette resulted in a recovery similar to that of PAO1. These data suggest that the presence of PC in the lipid bilayer of *P. aeruginosa* contributes to cell viability under these stress conditions.

DISCUSSION

The impact of lipid composition on bacterial growth is not sufficiently understood. Investigating lipid composition can have opposing focal points. On one hand, the process and regulation of synthesizing PC and other phospholipids can be delineated. On the other hand, one can focus on the phospholipid membrane as a target for phospholipid-hydrolyzing enzymes such as PLC and PLD that generate phospholipid-derived molecules involved in eukaryotic signaling. The impressive and quickly growing body of literature elucidating the involvement of phospholipids and phospholipid-derived molecules (e.g., DAG and phosphatidic acid) in cell signaling processes has generally focused on the effects of these signals relative to those in eukaryotic cells. In addition, the interest in phospholipid mediators has generally been limited to those produced by extracellular molecules, such as PLCs. Perhaps one reason for this focus on eukaryotic cells is that PC, one of the major targets for these phospholipid-hydrolyzing enzymes, is abundant in eukaryotic membranes.

Interestingly, *P. aeruginosa* is the only PC-containing bacterium identified thus far that also produces PC-hydrolyzing enzymes (e.g., PLC and PLD). Although *Yersinia pestis* also makes a PLD and encodes a homologue of PmtA, only the monomethyl-PE intermediate has been detected (35). *P. aeruginosa* produces several phospholipases that have been shown to be involved in virulence. For example, a hemolytic and a nonhemolytic PLC (23) have been shown to contribute to the pathogenesis of this opportunistic pathogen in several experimental models in animals, as well as in plants (24, 26). In addition, we recently identified a PLD gene like that of eukaryotes (*pldA*) and PldA has been shown to contribute to the pathogenesis of *P. aeruginosa* in the rat lung model of infection (37). Like these PLCs, PldA hydrolyzes PC; however, unlike with these secreted PLCs, PldA is localized to the periplasm (37). One function of the secreted PLCs in virulence is to hydrolyze the lipid membranes of eukaryotic cells. The role of PldA is less obvious due its localization to the periplasm, rendering it inaccessible to the eukaryotic PC. However, Albelo and Domenech (1) reported that when choline is used as the carbon source, PC is a normal component of *P. aeruginosa*, suggesting that PldA may be involved in lipid maintenance. We verified that this phospholipid is indeed in the membrane of *P. aeruginosa*, and we identified the Pcs pathway as being involved in its synthesis.

Using 2D-TLC, we verified by several methods that *P. aeruginosa* produces PC (Fig. 3). First, DAG was generated when a lipid comigrating with commercially available PC was extracted from PAO1 and digested with PlcH, a PC-specific PLC purified from *P. aeruginosa* PAO1 (Fig. 3). Second, analysis of the lipids using CID generated a product with a mass of 184 kDa, characteristic of PC (Fig. 4). In addition, we were able to identify the potential fatty acid side chains present, such as those listed in Table 2.

The consequences of PC production by a prokaryotic organism that itself produces phospholipid-hydrolyzing activities (e.g., PLC and PLD) suggest a potential role of these lipids and their by-products in signaling processes analogous to those known to occur in eukaryotes. Furthermore, one can envision that *P. aeruginosa* PLC activity must be regulated while it is inside the cell so as not to act on its own PC, subjecting itself to unwarranted signaling molecules. The PLD activity may also be regulated such that the PLD is involved in membrane maintenance and not in generating detrimental signaling molecules unless needed.

To determine their respective contribution to PC production, the *pmtA* and *pcs* homologues were cloned from *P. aeruginosa* PAO1. In both *E. coli* and *P. aeruginosa*, transformation with a vector containing *pcs* was sufficient for PC production while no PC was detected when the strains were transformed with a vector containing *pmtA*. Together, these and other data presented in this report suggest that, under these conditions, the methylation pathway using PmtA to generate PC is not functional in *P. aeruginosa* PAO1. Perhaps the difference in the N-terminal region of the *P. aeruginosa* PmtA reflects the evolution of a different function for this protein.

The exact role of the lipid composition on *P. aeruginosa* is unknown, but its elucidation may have significant impacts on understanding its different lifestyles. Thus far, the only phenotypic effect that we have observed in PC-deficient strains is their reduced recovery from storage in glycerol at −70°C. This result suggests that the composition of phospholipid membrane in *P. aeruginosa* may be important in specific responses to stress. The fact that *P. aeruginosa* can be found in diverse environments (soil, plants, human lung) in symbiotic and pathogenic relationships reiterates the necessity of the broad and redundant genetic armament that *P. aeruginosa* possesses. It is also intriguing that this organism produces both PC and PC-hydrolyzing enzymes.

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REFERENCES

1. **Albelo, S. T., and C. E. Domenech.** 1997. Carbons from choline present in the phospholipids of *Pseudomonas aeruginosa.* FEMS Microbiol. Lett. **156:**271–274.
2. **Arondel, V., C. Benning, and C. R. Somerville.** 1993. Isolation and functional expression in *Escherichia coli* of a gene encoding phosphatidylethanolamine methyltransferase (EC 2.1.1.17) from *Rhodobacter sphaeroides*. J. Biol. Chem. **268:**16002–16008.
3. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
4. **de Lorenzo, V., L. Eltis, B. Kessler, and K. N. Timmis.** 1993. Analysis of *Pseudomonas* gene products using *lacIq*/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. Gene **123:**17–24.
5. **De Meyer, G., K. Capieau, K. Audenaert, A. Buchala, J. P. Metraux, and M. Hofte.** 1999. Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. Mol. Plant-Microbe Interact. **12:**450–458.
6. **de Rudder, K. E., I. M. Lopez-Lara, and O. Geiger.** 2000. Inactivation of the gene for phospholipid *N*-methyltransferase in *Sinorhizobium meliloti*: phosphatidylcholine is required for normal growth. Mol. Microbiol. **37:**763–772.
7. **de Rudder, K. E., C. Sohlenkamp, and O. Geiger.** 1999. Plant-exuded choline is used for rhizobial membrane lipid biosynthesis by phosphatidylcholine synthase. J. Biol. Chem. **274:**20011–20016.
8. **de Rudder, K. E., J. E. Thomas-Oates, and O. Geiger.** 1997. *Rhizobium meliloti* mutants deficient in phospholipid *N*-methyltransferase still contain phosphatidylcholine. J. Bacteriol. **179:**6921–6928.
9. **Ferguson, M. W., J. A. Maxwell, T. S. Vincent, J. da Silva, and J. C. Olson.** 2001. Comparison of the *exoS* gene and protein expression in soil and clinical isolates of *Pseudomonas aeruginosa.* Infect. Immun. **69:**2198–2210.
10. **Figurski, D., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans.* Proc. Natl. Acad. Sci. USA **76:**1648–1652.
11. **Goldfine, H.** 1982. Lipids of procaryotes: structure and distribution. Curr. Top. Membr. Transp. **17:** 1–43.
12. **Haydock, S. F., J. A. Dowson, N. Dhillon, G. A. Roberts, J. Cortes, and P. F. Leadlay.** 1991. Cloning and sequence analysis of genes involved in erythromycin biosynthesis in *Saccharopolyspora erythraea*: sequence similarities between EryG and a family of S-adenosylmethionine-dependent methyltransferases. Mol. Gen. Genet. **230:**120–128.
13. **Hirakata, Y., B. B. Finlay, D. A. Simpson, S. Kohno, S. Kamihira, and D. P. Speert.** 2000. Penetration of clinical isolates of *Pseudomonas aeruginosa* through MDCK epithelial cell monolayers. J. Infect. Dis. **181:**765–769.
14. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:**73–102.
15. **Ingrosso, D., A. V. Fowler, J. Bleibaum, and S. Clarke.** 1989. Sequence of the D-aspartyl/L-isoaspartyl protein methyltransferase from human erythrocytes. Common sequence motifs for protein, DNA, RNA, and small molecule S-adenosylmethionine-dependent methyltransferases. J. Biol. Chem. **264:**20131–20139.
16. **Kaneshiro, T., and J. H. Law.** 1964. Phosphatidylcholine synthesis in *Agrobacterium tumefaciens*. I. Purification and properties of a phosphatidylethanolamine *N*-methyltransferase. J. Biol. Chem. **239:**1705–1713.
17. **Kennedy, E. P.** 1989. Discovery of the pathways for the biosynthesis of phosphatidylcholine, p. 1–9. *In* D. E. Vance (ed.), Phosphatidylcholine metabolism. CRC Press, Boca Raton, Fla.
18. **Lopez-Lara, I. M., and O. Geiger.** 2001. Novel pathway for phosphatidylcholine biosynthesis in bacteria associated with eukaryotes. J. Biotechnol. **91:**211–221.
19. **Lui, P.** 1966. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. II. Effects of lecithinase and protease. J. Infect. Dis. **16:**112–116.
20. **Minder, A. C., K. E. de Rudder, F. Narberhaus, H. M. Fischer, H. Hennecke, and O. Geiger.** 2001. Phosphatidylcholine levels in *Bradyrhizobium japonicum* membranes are critical for an efficient symbiosis with the soybean host plant. Mol. Microbiol. **39:**1186–1198.
21. **Mizuno, T., and M. Kageyama.** 1978. Separation and characterization of the outer membrane of *Pseudomonas aeruginosa*. J. Biochem. (Tokyo) **84:**179–191.
22. **Nunn, D., S. Bergman, and S. Lory.** 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J. Bacteriol. **172:**2911–2919.
23. **Ostroff, R. M., A. I. Vasil, and M. L. Vasil.** 1990. Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from *Pseudomonas aeruginosa*. J. Bacteriol. **172:**5915–5923.
24. **Ostroff, R. M., B. Wretlind, and M. L. Vasil.** 1989. Mutations in the hemolytic-phospholipase C operon result in decreased virulence of *Pseudomonas aeruginosa* PAO1 grown under phosphate-limiting conditions. Infect. Immun. **57:**1369–1373.
25. **Raetz, C. R., and W. Dowhan.** 1990. Biosynthesis and function of phospholipids in *Escherichia coli.* J. Biol. Chem. **265:**1235–1238.
26. **Rahme, L. G., E. J. Stevens, S. F. Wolfort, J. Shao, R. G. Tompkins, and F. M. Ausubel.** 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science **268:**1899–1902.
27. **Rahme, L. G., M. W. Tan, L. Le, S. M. Wong, R. G. Tompkins, S. B. Calderwood, and F. M. Ausubel.** 1997. Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. Proc. Natl. Acad. Sci. USA **94:**13245–13250.
28. **Rock, R. O., S. Jackowski, and J. E. Cronan.** 1996. Lipid metabolism in prokaryotes, p. 35–74. *In* D. E. Vance and J. E. Vance (ed.), Biochemistry of lipids, lipoproteins and membranes. Elsevier, Amsterdam, The Netherlands.
29. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
31. **Simon, R., U. Priefer, and A. Puehler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–791.
32. **Stanislavsky, E. S., T. A. Makarenko, and T. E. Kozhenova.** 1992. Specific and nonspecific mouse protection induced by different chemotypes of the *Pseudomonas aeruginosa* lipopolysaccharide. FEMS Microbiol. Immunol. **105:**181–190.
33. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, and I. T. Paulsen.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959–964.
34. **Tang, Y., and R. I. Hollingsworth.** 1998. Regulation of lipid synthesis in *Bradyrhizobium japonicum*: low oxygen concentrations trigger phosphatidylinositol biosynthesis. Appl. Environ. Microbiol. **64:**1963–1966.
35. **Tornabene, T. G.** 1973. Lipid composition of selected strains of *Yersinia pestis* and *Yersinia pseudotuberculosis*. Biochim. Biophys. Acta **306:**173–185.
36. **Vasil, M. L., C. Chamberlain, and C. C. Grant.** 1986. Molecular studies of *Pseudomonas* exotoxin A gene. Infect. Immun. **52:**538–548.
37. **Wilderman, P. J., A. I. Vasil, Z. Johnson, and M. L. Vasil.** 2001. Genetic and biochemical analyses of a eukaryotic-like phospholipase D of *Pseudomonas aeruginosa* suggest horizontal acquisition and a role for persistence in a chronic pulmonary infection model. Mol. Microbiol. **39:**291–304.
38. **Wilderman, P. J., A. I. Vasil, Z. Johnson, M. J. Wilson, H. E. Cunliffe, I. L. Lamont, and M. L. Vasil.** 2001. Characterization of an endoprotease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa*. Infect. Immun. **69:**5385–5394.
39. **Wilkinson, S. G.** 1988. Gram-negative bacteria, p. 299–488. *In* C. Ratledge and S. G. Wilkinson (ed.), Microbial lipids, vol. 1. Academic Press Inc., San Diego, Calif.
40. **Williams, J. G., and C. R. McMaster.** 1998. Scanning alanine mutagenesis of the CDP-alcohol phosphotransferase motif of *Saccharomyces cerevisiae* cholinephosphotransferase. J. Biol. Chem. **273:**13482–13487.