The most widely distributed biosynthetic pathway to initiate phosphatidic acid formation in bacterial membrane phospholipid biosynthesis involves the conversion of acyl-acyl carrier protein to acylphosphate by PlsX and the transfer of the acyl group from acylphosphate to glycerol-3-phosphate by an integral membrane protein, PlsY. The membrane topology of Streptococcus pneumoniae PlsY was determined using the substituted cysteine accessibility method. PlsY has five membrane-spanning segments with the amino terminus and two short loops located on the external face of the membrane. Each of the three larger cytoplasmic domains contains a highly conserved sequence motif. Site-directed mutagenesis revealed that each conserved domain was critical for PlsY catalysis. Motif 1 had an essential serine and arginine residue. Motif 2 had the characteristics of a phosphate-binding loop. Mutations of the conserved glycines in motif 2 to alanines resulted in a defect for glycerol-3-P binding leading to the conclusion that this motif corresponds to the glycerol 3-phosphate binding site. Motif 3 contained a conserved histidine and asparagine that were important for activity and a glutamate that was critical to the structural integrity of PlsY. PlsY was noncompetitively inhibited by palmitoyl-CoA. These data define the membrane architecture and the critical active site residues in the PlsY family of bacterial acyltransferases.

PtOH is the key intermediate in bacterial phospholipid biosynthesis (for reviews see Refs. 1 and 2). PtOH is synthesized by the successional acylation of glycerol-3-P, and there are two routes to 1-acylglycerol-3-P. In the Escherichia coli model system, either acyl-ACP or acyl-CoA thioesters are utilized by the membrane-bound PlsB acyltransferase to acylate the 1-position of glycerol-3-P (1, 3, 4) (Fig. 1). However, the E. coli paradigm is not universal, and the plsB gene is confined to Gram-negative bacteria (primarily γ-proteobacteria) (5). The important human Gram-positive pathogens utilize the recently discovered PlsX/Y pathway for 1-acylglycerol-3-P formation (5). PlsX is a soluble protein that catalyzes the formation of acyl-PO4 from acyl-ACP. This activated fatty acid is then used by the membrane-bound PlsY acyltransferase to acylate the 1-position of glycerol-3-P (Fig. 1). The second acyltransferase in PtdOH formation, PlsC, is universally expressed in bacteria and completes the synthesis of PtdOH by transferring an acyl chain from acyl-ACP (acyl-CoA) to the 2-position of 1-acylglycerol-3-P (5) (Fig. 1).

The plsB gene was identified in 1974 with the isolation of E. coli strain BB26, a glycerol-3-P auxotroph that has a missense mutation that results in an elevated Km for glycerol-3-P (6–8). PlsB is responsible for the selection of fatty acids incorporated into membrane phospholipids and is a key regulatory point in the pathway (1, 9, 10). PlsB protein of bacteria and mice has been investigated extensively, and there is a considerable amount of information available on its membrane topology, phospholipid requirements, and active site residues (4, 11–19). PlsY represents a unique glycerol-3-P acyltransferase superfamily of proteins that is essential for membrane biogenesis in many bacteria (5). It is much smaller than PlsB (23 versus 93 kDa) and lacks the characteristic acyltransferase amino acid sequence motifs found in the bacterial and mammalian PlsB and PlsC acyltransferases (17, 18). The goal of this study was to define the membrane topology and the amino acid sequence motifs required for PlsY activity. We used the PlsY of Streptococcus pneumoniae as the prototypical protein to study this widely expressed and highly conserved family of membrane acyltransferases.

EXPERIMENTAL PROCEDURES

Materials—Sources of supplies were as follows: Avanti Polar Lipids, Inc. supplied phospholipids; Sigma, glycerol-3-P and fatty acid-free bovine serum albumin; Santa Cruz Biotechnology Inc., anti-His antibody; Molecular Probes, N-(3-maleimido-propionyl)biocytin (MPB); Pierce, streptavidin alkaline phosphatase; American Radiolabeled Chemicals Inc., sn-[U-14C]glycerol 3-phosphate (specific activity, 150 mCi/mmol); Fisher, Whatman 3MM filter paper; Stratagene, QuiKChange site-directed mutagenesis kit; Promega, restriction enzymes. Protein was measured by the Bradford method (20), and 16:0-P04 was synthesized as described previously (5). All other chemicals were of reagent grade or better.

Preparation and Assay of Aciyltransferase Activity—E. coli membranes were purified and acyltransferase activity meas-
Topo
logy and Active Site of PlsY

FIGURE 1. Pathways for PtdOH synthesis in bacteria. There are two routes to acylglycerol-3-P. The major pathway in bacteria uses soluble PlsX to convert the acyl-ACP end-products of fatty acid synthesis to acyl-PO₄, followed by the membrane-associated PlsY to transfer the acyl moiety to the 1-position of glycerol-3-P. In some bacteria like E. coli, glycerol-3-P is acylated by PlsB, a membrane-bound enzyme that utilizes either acyl-ACP or acyl-CoA as the acyl donor. Acylation of the 2-position is catalyzed by PlsC, a universally expressed, membrane-bound 1-acylglycerol-3-P acyltransferase that predominantly uses acyl-ACP, although some PlsCs also use acyl-CoA.

ured as described previously (5). Briefly, the reaction buffer contained 100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mg/ml bovine serum albumin, 5 mM Na₃VO₄ (to inactive phosphatases), and purified membranes. 16:0-PO₄ was added to start the reaction. Reactions were terminated after at 37 °C for 30 min by pipetting 20 µl of the reaction mixture onto a Whatman 3MM cellulose filter disc. Filter discs were washed in 10, 5, and 1% ice-cold trichloroacetic acid (for 20 min, 20 ml/disc) prior to scintillation counting (21).

The activities of the mutants were compared with the wild-type protein as described by Zhang et al. (22). Aliquots contain-
acetic acid beads and incubated at room temperature for 2 h with shaking. The beads were washed once with the same buffer and twice with buffer containing 40 mM imidazole and then eluted with buffer adjusted to 200 mM imidazole. Samples were analyzed by SDS-PAGE followed by immunoblotting using anti-His antibody and streptavidin-AP conjugate following standard protocol.

Kinetic Analysis—The kinetic parameters for glycerol-3-P and 16:0-PO_4 were obtained by linear regression analysis of double reciprocal plot using Prism 4 (GraphPad Software). The \( K_m \) for glycerol-3-P was determined by fixing the 16:0-PO_4 concentration to 200 \( \mu M \) while varying the [\(^{14}\)C]glycerol-3-P concentration from 50 to 800 \( \mu M \). The \( K_m \) for 16:0-PO_4 was obtained by varying the concentration of 16:0-PO_4 from 3.125 to 50 \( \mu M \) at a fixed [\(^{14}\)C]glycerol-3-P concentration of 200 \( \mu M \).

RESULTS

Membrane Topology of PlsY—PlsY was associated with the membrane fraction of \textit{S. pneumoniae} or \textit{E. coli} cells expressing PlsY when acylphosphate acyltransferase activity was assayed using fractions separated by sucrose gradient ultracentrifugation (5). This behavior was characteristic for an integral membrane protein; thus we performed a hydrophathy analysis of PlsY using the Kyte-Doolittle algorithm with a window size of 19 residues to predict the transmembrane domains in PlsY (25). The prediction did not clearly demonstrate a pattern of hydrophobicity that allowed us to confidently define the membrane topology by visual inspection (Fig. 2A). The analysis of the PlsY sequence using two membrane protein structure prediction programs, SOSUI (26) and TMHMM (27), showed that PlsY contained several possible transmembrane domains; however, the programs did not agree on the topology. Both models predicted the existence of significant protein loops that contained highly conserved amino acid sequences exposed to cell exterior. Because the substrates for PlsY are generated on the cytoplasmic side of the membrane, it did not seem reasonable that an extended loop with a highly conserved sequence motif would be found in the extracellular compartment.

The SCAM approach, based on the controlled membrane permeability of sulfhydryl reagents, was used to directly determine the topology of PlsY (23, 24). Single Cys replacement mutants at different locations along the PlsY sequence (Fig. 2B) were introduced, and the modified PlsYs were expressed as carboxyl-terminal His-tagged proteins in \textit{E. coli}. MPB is impermeable to the inner membranes of \textit{E. coli} (28). The presence of toluene in the solution permeabilized the membrane and allowed MPB to biotinylate cysteines on the cytoplasmic side of the membrane. There was one cysteine in the wild-type PlsY sequence at position 121. This cysteine was used in our analysis, but Cys-121 was mutated to serine for the analysis of the other constructs. Membranes prepared from \textit{E. coli} expressing PlsY and PlsY[C121S] had same amount of PlsY as detected by immunoblot using anti-His antibody and showed no difference in their specific PlsY acyltransferase activity, illustrating that removal of Cys-121 did not affect the activity or membrane targeting of PlsY. We selected residues spaced along the PlsY sequence, postulated as being in the loop regions based on hydrophathy analysis (Fig. 2A), and mutated them to cysteine in the PlsY[C121S] background. \textit{E. coli} cells expressing these proteins were first treated with 10 mM dithiothreitol to ensure that all the exposed cysteines were in the reduced form. Dithiothreitol was then removed by washing with phosphate-buff ered saline, and the cells were labeled with 100 \( \mu M \) MPB in the presence or absence of toluene. PlsY[I2C], PlsY[H75C], and PlsY[I162C] were labeled by MPB in the absence of toluene, which placed these three residues in the extracellular space (Fig. 2B). PlsY[T39C], PlsY[K51C], PlsY[A105C], wild-type PlsY(C121), PlsY[G135C], and PlsY[A190C] showed tolutene-dependent MPB labeling, which placed these residues in the cytoplasm (Fig. 2B). The same amount of each PlsY mutant protein was present in each experiment as indicated by immunoblot analysis using anti-His antibody (Fig. 2B). Two other mutants (PlsY[I59C] and PlsY[I90C]) were generated that did not label with MPB either in the presence of absence of toluene (not shown). These results suggest that these residues were likely located in the membrane-spanning regions of PlsY and were not accessible to MPB either in the presence or absence of toluene (23, 24). Based on these data, the hydrophathy analysis and a length of 22 residues for a transmembrane segment, the five membrane-spanning segments of PlsY were predicted to be located between residues 5–27, 52–74, 78–99, 139–161, and 163–184, three short regions exposed to the outside of the cell, and three cytoplasmic domains. However, the precise cutoff for the beginning end of each transmembrane domain cannot be determined from this data.

There were no ambiguous results in the SCAM analysis, which indicated that \( \geq 97\% \) of the PlsY was in the same orientation in the membrane. The level of PlsY overexpression in the experimental system was modest. The specific activity of PlsY in \textit{S. pneumoniae} membranes was 3.7 ± 0.1 pmol/min/\( \mu g \). The PlsY specific activity in the \textit{E. coli} expression system was 18.28 ± 1.49 pmol/min/\( \mu g \), which was only 5-fold higher than found in native \textit{S. pneumoniae} membranes. The topology is also consistent with the finding that amino-terminal His-tagged PlsY did not assemble into the membrane. This was attributed to the inability to move the charged His tag through
the bilayer to the extracellular face. This is why we used carboxyl-terminal His-tagged PlsY for all of our experiments.

**Kinetic Constants for PlsY**—The activity of PlsY did not require divalent or monovalent cations, and the addition of 1 mM EDTA to the reaction did not affect its activity either. PlsY was active between pH 6.5 and 9.5 with maximal activity at pH 7.4 (data not shown). We determined the $K_m$ values for the two substrates of PlsY, glycerol-3-P and 16:0-PO$_4$, using purified membranes from *E. coli* expressing *S. pneumoniae* PlsY. The apparent $K_m$ for glycerol-3-P was 100 μM (Fig. 3A) and for 16:0-PO$_4$ was 30 μM (Fig. 3B), as calculated from the double reciprocal plot. These $K_m$ values are comparable with those of PlsB for glycerol-3-P and 16:0-CoA, which are 90 and 20 μM, respectively (9).

**Three Conserved Sequence Motifs in PlsY**—We aligned 417 PlsY sequences found in the Integrated Genomic Bacterial Database and identified three regions containing completely conserved amino acid sequences (Fig. 4). All three motifs were located in the cytoplasmic surface of PlsY based on the topology determination, and the high degree of conservation of these sequences suggests that they are critical to catalysis. Site-directed mutagenesis was employed to analyze the effect of altering these conserved residues on PlsY activity with the goal of ascribing functions to each of the domains in this new acyltransferase superfamily of proteins. Each mutant protein was expressed in *E. coli* strain FB23281 (*plsY::Tn5*) to ablate background activity because of the low level of endogenous PlsY expression. The expression and assembly into membranes of each of the mutants was assessed by immunoblot analysis, which detected the carboxyl-terminal His tag in PlsY in membranes purified from cells expressing the mutant proteins. The biochemical activity of these mutant PlsY proteins in *E. coli* membranes was normalized to the amount of protein expression based on the Western blots (Fig. 5), analyzed, and compared with wild-type PlsY expression as described under “Experimental Procedures” (Table 1). Proteins scored as inactive had <0.05% of wild-type activity. This lower limit of detection was established from the highest amount of membrane protein that could be added to the assay without our detecting product formation.

Motif 1 (residues 35–46) was defined by the consensus sequence GSGNXGXTNXXR and was located in the first cytoplasmic loop (Fig. 4). This glycine-rich region was reminiscent of phosphate-binding loops found in other proteins, and mutation of the single positively charged residue in this cluster (PlsY[R46A]) resulted in an inactive enzyme (Table 1). Also, Ser-36 was essential for activity. Mutations of the other conserved residues gave rise to kinetically compromised enzymes that retained some activity. Kinetic analysis of these mutants showed that the substrate $K_m$ values were less than 2-fold different from the wild type, with the main effect being in the maximum velocity of the reaction (not shown).

Motif 2 (residues 100–107) was defined by the sequence FXGKKXVA and was located on the second cytoplasmic loop (Fig. 4). Lys-104 was essential for catalysis in this cluster as indicated by the lack of activity in the PlsY[K104A] mutant (Table 1). Lys-101 and Ala-105 were not highly conserved residues, and accordingly, the PlsY[K101A] and PlsY[A105C] mutants had wild-type activity. Mutation of the highly conserved glycines by the addition of a methyl side chain had severe consequences for catalysis, although the proteins retained some acyltransferase activity.

The sequence of motif 2 reflected the properties of known phosphate-binding loops that have a positively charged residue adjacent to one or two glycines (29, 30). The glycine residues are conserved in these structures because the presence of side chains would sterically block access to the substrate binding pocket. Therefore, we analyzed the catalytically defective
PlsY[G102A] and PlsY[G103A] mutants for $K_m$ defects in substrate binding. There was no difference in the 16:0-PO$_4$ $K_m$ between the two mutant proteins and PlsY (Fig. 6A). However, the $K_m$ for glycerol-3-P increased from 92 $\mu$M in PlsY to 670 $\mu$M.

Three highly conserved intracellular sequence motifs were clearly evident. Residues with a frequency of occurrence over 75% are highlighted in black in the S. pneumoniae PlsY sequence shown below the graphical analysis. Motif 1 was located in the first cytoplasmic loop, motif 2 was located in the second cytoplasmic loop, and motif 3 was located in the carboxyl-terminal cytoplasmic domain.

### TABLE 1

| Protein | Specific activity$^a$ (pmol/min/μg) | Activity % |
|---------|------------------------------------|------------|
| PlsY    | 18.28 ± 1.49                      | 100        |
| **Motif 1** |                                   |            |
| PlsY[S36A] | <0.01$^b$                         | <0.05      |
| PlsY[S36C] | <0.01                              | <0.05      |
| PlsY[N38A] | 10.75 ± 0.36                      | 58.8       |
| PlsY[T39C] | 10.23 ± 0.67                      | 56.0       |
| PlsY[T42A] | 22.43 ± 0.84                      | 122.7      |
| PlsY[N43A] | 0.85 ± 0.08                        | 5.1        |
| PlsY[R46A] | <0.01                             | <0.05      |
| PlsY[R66S] | <0.01                             | <0.05      |
| **Motif 2** |                                   |            |
| PlsY[K101A] | 17.67 ± 0.41                      | 96.7       |
| PlsY[G102A] | 1.70 ± 0.18                       | 9.5        |
| PlsY[G103A] | 0.22 ± 0.02                       | 1.2        |
| PlsY[K104A] | <0.01                             | <0.05      |
| PlsY[K104S] | <0.01                             | <0.05      |
| PlsY[A105C] | 16.37 ± 1.11                       | 89.6       |
| **Motif 3** |                                   |            |
| PlsY[H185A] | 0.11 ± 0.01                       | 0.6        |
| PlsY[K186A] | 9.71 ± 0.61                       | 53.1       |
| PlsY[N188A] | <0.01                             | <0.05      |
| PlsY[E197A] | _                                 | _          |

- $^a$ Plasmid vectors expressing S. pneumoniae PlsY mutant proteins were transformed into E. coli strain FB23281 (plsY::Tn5). Membranes from these strains were prepared, the acyltransferase assays were performed, and the activities were normalized based on the level of mutant protein expression compared to wild-type PlsY as described under “Experimental Procedures.”
- $^b$ The lower limit of detection in the assay was 0.05% of wild-type PlsY activity or 0.01 pmol/min/μg.
- $^c$ PlsY[E197A] was not detected in the membrane by immunoblotting, and thus the activity of this protein was <0.01 pmol/min/μg.

PlsY[G102A] and PlsY[G103A] mutants for $K_m$ defects in substrate binding. There was no difference in the 16:0-PO$_4$ $K_m$ between the two mutant proteins and PlsY (Fig. 6A). However, the $K_m$ for glycerol-3-P increased from 92 $\mu$M in PlsY to 670 $\mu$M.
in both of the mutants (Fig. 6B). These data support the conclusion that motif 2 corresponded to the glycerol-3-P substrate binding site.

Motif 3 (residues 185–197) was defined by the sequence HX₂NX₉E and was located in the carboxyl-terminal cytoplasmic domain. Histidine plays an important role in the PlsB acyltransferase reaction by acting as a general base to abstract a proton from the hydroxyl group of the acyl acceptor (21). There were two conserved histidines in PlsY. His-92 was found in a majority of PlsY sequences; however, PlsY[H92A] assembled and was as active as the wild-type protein (not shown). This result was consistent with our topology experiments that placed His-92 as a component of a transmembrane helix. In contrast, PlsY[H185A] was catalytically defective (Table 1). Asn-188 was an essential residue in motif 3 and was required for acyltransferase activity. PlsY[E197A] was also inactive; however, this protein was not detected in immunoblots. This finding suggests that this highly conserved glutamate is important to protein folding and/or membrane assembly rather than being directly involved in catalysis.

**Acyl-CoA Inhibition of PlsY**—Our previous experiments with *S. pneumoniae* membranes showed that long chain (16:0) acyl-CoA reduced the incorporation of 16:0-PO₄ into acylglycerol-3-P. We examined the mechanism for 16:0-CoA inhibition of PlsY by expressing *plsY* in strain SJ361 (*plsB26 plsX50*...
that are involved in catalysis and substrate binding (18, 21). Prototypical acyltransferases, exemplified by PlsB and PlsC, are essential for catalysis. There are four conserved blocks of amino acids in the PlsY protein family that are crucial for catalysis. The motifs within the PlsY protein family that are essential for catalytic activity (Figs. 4 and 8). Motif 2 in the second cytoplasmic loop was proposed as the glycerol-3-P binding site based on the glycerol-3-P Km defects associated with the PlsY[G102A] and PlsY[G103A] mutants. The ATP binding sites of prototypical kinases have so-called phosphate-binding loops, which consist of a glycine-rich sequence followed by a lysine residue (29, 30). The glycines are important because the presence of side chains would interfere with substrate binding, consistent with the Km defects we observed in the PlsY[G102A] and PlsY[G103A] mutants. Positively charged side chains are important for phosphate binding in enzymes (31), and Arg-354 of PlsB is an essential component of the glycerol-3-P substrate binding pocket (18). Motif 2 contains Lys-104 downstream of the glycines, which is clearly essential for activity and may be a key player in binding the phosphate of glycerol-3-P. The site-directed mutants in motif 1 located in the first cytoplasmic loop did not provide a clear picture concerning its function in catalysis. It is obvious that Arg-46 is essential for catalysis and that Asn-43 is important. An NXXR motif acts as the γ-phosphate-binding motif in the Hsp90-ATP complex (32), suggesting a similar phosphate binding role for motif 1. Thus, motif 1 may be critical for the binding of acyl-PO4 based on the assignment of glycerol-3-P binding to motif 2. Ser-36 is another essential residue in motif 1. We do not know the role of this residue, although one idea that should be tested is that PlsY functions through an acyl-enzyme intermediate. Motif 3 is in the carboxyl-terminal cytoplasmic segment and contains a conserved histidine that contributes to catalysis based on the compromised activity of the PlsY[H185A] mutation. The proposed role of His-185 is that same as the proposed role of the histidine in the HX4D motif found in PlsB and other acyltransferases (18, 21). Conserved histidines in acyltransferases act as a general base to activate the glycerol-3-P hydroxyl to attack either acyl-ACP or acyl-CoA. His-185 in motif 3 may also act as a general base to abstract a proton from the hydroxy group of glycerol-3-P to facilitate the nucleophilic attack on the phosphoanhydride bond of acylphosphate. The conserved glutamate in motif 3 likely functions in protein folding, because the PlsY[E197A] mutant failed to assemble into the membrane.

The mechanisms that control the activity of the PlsX/Y pathway are poorly understood, but our work provides some insight into how PlsB and PlsY functions are coordinates in bacteria that express both types of acyltransferases. PlsY is noncompetitively inhibited by long chain acyl-CoA. This regulatory property is understood within the context of bacteria such as E. coli...
that import fatty acids from the environment and convert them to acyl-CoA derivatives. These acyl-CoAs are not only used for β-oxidation but also are incorporated into membrane phospholipid by PbsB, therefore reducing the demand for acyl moieties derived from fatty acid synthesis. Acyl-CoA inhibition of PlsY contributes by shutting down the use of acyl moieties from fatty acid biosynthesis (acyl-ACP) when an alternate source of fatty acid is present. Genetic regulation of the pathway is more puzzling. The global transcriptional regulator FapR in \textit{B. subtilis} regulates \textit{plscX} and \textit{plscC} expression but not \textit{plspY} (33). However, in \textit{S. pneumoniae} fatty acid synthesis genes and \textit{plscC} are regulated by the FabT transcriptional repressor, but \textit{plscX} and \textit{plspY} are not (34). The importance of these differences in transcriptional regulation in bacteria that rely on the PlsX/Y pathway as the sole route to membrane phospholipids remains a significant avenue of exploration.

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