Published in final edited form as:

Mol Microbiol. 2014 April ; 92(2): 234–245. doi:10.1111/mmi.12556.

Incorporation of Extracellular Fatty Acids by a Fatty Acid Kinase-Dependent Pathway in *Staphylococcus aureus*

Joshua B. Parsons, Matthew W. Frank, Pamela Jackson, Chitra Subramanian, and Charles O. Rock*

Department of Infectious Diseases, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105

Summary

Acyl-CoA and acyl-acyl carrier protein (ACP) synthetases activate exogenous fatty acids for incorporation into phospholipids in Gram-negative bacteria. However, Gram-positive bacteria utilize an acyltransferase pathway for the biogenesis of phosphatidic acid that begins with the acylation of *sn*-glycerol-3-phosphate by PlsY using an acyl-phosphate (acyl-PO₄) intermediate. PlsX generates acyl-PO₄ from the acyl-ACP end-products of fatty acid synthesis. The *plsX* gene of *Staphylococcus aureus* was inactivated and the resulting strain was both a fatty acid auxotroph and required *de novo* fatty acid synthesis for growth. Exogenous fatty acids were only incorporated into the 1-position and endogenous acyl groups were channeled into the 2-position of the phospholipids in strain PDJ39 (*plsX*). Extracellular fatty acids were not elongated. Removal of the exogenous fatty acid supplement led to the rapid accumulation of intracellular acyl-ACP and the abrupt cessation of fatty acid synthesis. Extracts from the *plsX* strain exhibited an ATP-dependent fatty acid kinase activity, and the acyl-PO₄ was converted to acyl-ACP when purified PlsX is added. These data reveal the existence of a novel fatty acid kinase pathway for the incorporation of exogenous fatty acids into *S. aureus* phospholipids.

Keywords

fatty acid synthesis; PlsX; PlsY; acyl carrier protein; acyl-phosphate; *S. aureus*

Introduction

Phosphatidic acid (PtdOH) is a universal intermediate in the biosynthesis of membrane phospholipids in eubacteria (Yao and Rock, 2013). In the *Escherichia coli* model, PtdOH formation is initiated by the acylation of *sn*-glycerol-3-phosphate (G3P) by PlsB, a G3P acyltransferase. PlsB of *E. coli* has been extensively characterized and utilizes either acyl-acyl carrier protein (ACP) or acyl-CoA thioesters to acylate the 1-position of G3P (Rock *et al.*, 1981a; Lightner *et al.*, 1980; Green *et al.*, 1981). PlsB is responsible for the selection of fatty acids incorporated into the 1-position of membrane phospholipids and is a key regulatory point in the pathway (Yao and Rock, 2013; Rock *et al.*, 1981b; Cronan, Jr. and Rock, 1996; Heath *et al.*, 1994). However, most bacteria, including important human Gram-

*For correspondence. charles.rock@stjude.org; Phone: (901) 595-3491; Fax: (901) 595-3099.

positive pathogens such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, lack a *plsB* gene and instead use the PlsX/PlsY pathway for the acylation of G3P (Lu *et al.*, 2006) (Fig. 1). In these systems, the soluble PlsX is an acyl-ACP:PO₄ transacylase that converts the acyl-ACP end-products of *de novo* fatty acid synthesis to their acyl-PO₄ derivatives. These activated fatty acids are then used by the integral membrane protein PlsY to acylate G3P (Lu *et al.*, 2006; Lu *et al.*, 2007). A second acyltransferase, PlsC (Coleman, 1992), is universally expressed in bacteria and completes the synthesis of PtdOH by transferring a fatty acid to the 2-position of acyl-G3P. The *E. coli* PlsC uses either acyl-ACP or acyl-CoA as acyl donors, but the Gram-positive PlsCs use only acyl-ACP as substrate (Lu *et al.*, 2006; Yao and Rock, 2013).

Exogenous fatty acids can access these acyltransferase systems after their uptake by the cell and activation. In *E. coli*, the pathway involves the conversion of the fatty acid to an acyl-CoA thioester by FadD, an acyl-CoA synthetase (Yao and Rock, 2013). *E. coli* acyltransferases use acyl-CoAs as substrates, but this organism cannot convert fatty acids or acyl-CoAs to acyl-ACP. Thus, there is no elongation of exogenous fatty acids by FASII or incorporation into lipopolysaccharide via the acyl-ACP-specific acyltransferases. However, some bacteria possess an acyl-ACP synthetase that ligates fatty acids to ACP. The expression of this enzyme allows exogenous fatty acids to not only be used by the acyltransferases, but also to enter the fatty acid biosynthetic pathway and be elongated (Jiang *et al.*, 2006; Jiang *et al.*, 2010). Our recent work with *Staphylococcus aureus* shows that exogenous fatty acids are both used by the acyltransferases and elongated by FASII showing that they are converted to acyl-ACP (Parsons *et al.*, 2011). Experiments with crude extracts showed the formation of acyl-ACP from a labeled fatty acid, ATP and Mg²⁺, but the gene encoding the putative acyl-ACP synthetase was not identified.

The goal of this study is to determine if exogenous fatty acids are taken up in *S. aureus* via an acyl-ACP or acyl-PO₄ dependent pathway (Fig. 1). The key to distinguishing these two possibilities is the phenotype of a *plsX* deletion mutant. If fatty acids are activated to acyl-ACPs, then a *plsX* strain would be non-viable because they would be unable to synthesize acyl-PO₄ from either exogenous fatty acids or FASII. However, if a fatty acid kinase pathway is operating, a *plsX* strain would be able to produce acyl-PO₄ from exogenous fatty acids for PlsY and obtain acyl-ACP for PlsC from *de novo* fatty acid synthesis. PlsX is considered an essential gene based on the growth phenotype of a *Bacillus subtilis* strain conditionally deficient in *plsX* expression (Paoletti *et al.*, 2007). However, the media used in these experiments was not supplemented with fatty acids. We find *Staphylococcus aureus* *plsX* strains are both fatty acid auxotrophs and require *de novo* fatty acids synthesis for growth. Exogenous fatty acids are not elongated in PlsX-null *S. aureus*, and cell extracts exhibit fatty acid kinase activity. Thus, the incorporation of host fatty acids into the phospholipids of Gram-positive pathogens involves a novel fatty acid kinase to activate exogenous fatty acids for incorporation into phospholipids (Fig. 1).

Results

As previously reported (Parsons *et al.*, 2011), we found 100 μ M oleate inhibited [¹⁴C]acetate incorporation into *S. aureus* strain RN4220 (Fig. 2A). Oleate was used in most of our

experiments as the prototypical exogenous fatty acid because its incorporation and metabolism was easily separated from the FASII-produced saturated branched-chain fatty acids, and oleate is an abundant host fatty acid. The reduction in FASII activity was accompanied by incorporation of the exogenous oleate (18:1 9), and its elongation product 20:1 11, into phospholipids replacing the normal branched-chain saturated fatty acids. *S. aureus* lacks the capacity for β -oxidation and there were no [^{14}C]acetate-labeled fatty acids detected in the medium in these experiments. The regulatory effect of exogenous fatty acid was not a unique property of oleate (Fig. 2A). Lauric acid, a mixture of *anteiso* 15:0/17:0, or 17:0 also reduced the amount of [^{14}C]acetate labeling. Neither exogenous short-chain fatty acids (octanoic acid) nor very-long chain fatty acids (19:0 and 21:0) reduced the rate of acetate incorporation, suggesting the most highly incorporated fatty acids were acyl chains that were normally found in phospholipids. These data suggested that *S. aureus* was able to spare some energy expended in fatty acid synthesis when exogenous fatty acids are available.

In *E. coli*, acyl-CoA synthetase (FadD) plays a key role in the incorporation of exogenous fatty acids by generating the acyl-CoA substrates for the PlsB/PlsC acyltransferases (DiRusso *et al.*, 1999). There was no apparent role for acyl-CoA in phospholipid synthesis in bacteria that employ the acyl- PO_4 /acyl-ACP-dependent PlsX/PlsY/PlsC acyltransferase system (Lu *et al.*, 2006). We did not detect acyl-CoA synthetase activity in *S. aureus* extracts (not shown). There were two open reading frames in the *S. aureus* genome annotated as potential acyl-CoA synthetases, SA0226 and SA0533, which have 23% and 28% identity with FadD, respectively. These two genes were inactivated and the resulting strains did not have a deficiency in exogenous fatty acid incorporation into phospholipids as assessed by [^{14}C]oleate labeling compared to the parent strain RN4220 (not shown). Oleoyl-CoA was not detected in *S. aureus* strain RN4220 grown in the presence of exogenous oleate using ESI-MS/MS (not shown).

To validate these negative results, we inserted the *E. coli fadD* gene into plasmid pG164, which together with strain SA178R1, constituted a tightly-regulated *S. aureus* expression system (D'Elia *et al.*, 2006). Strain SA178R1 was derived from strain RN4220 and have the T7 polymerase and the LacI^q repressor genes integrated into the chromosome under the control of the *Pspac* promoter/operator allowing IPTG-dependent expression from the pG164 plasmid. The addition of 200 μM oleic acid did not affect the growth of strain SA178R1/pG164 either in the presence or absence of inducer (Fig. 2B). When *fadD* expression was induced by IPTG in strain SA178R1/pJP106(*fadD*), the presence of oleic acid caused a significant decrease in growth rate, and there was a slight effect on growth in the absence of induction (Fig. 2B). These data suggested that the formation of oleoyl-CoA in the strain expressing FadD had a deleterious effect. We have not explored the basis for the growth inhibition by FadD expression, but it is known that acyl-CoAs are potent inhibitors of the acyl- PO_4 -dependent PlsY acyltransferase (Lu *et al.*, 2006), and they may have inhibitory effects on other enzymes. Oleoyl-CoA was not detected in IPTG-treated strain SA178R1/pG164 exposed to oleic acid (Fig. 2C), consistent with our preliminary experiments with strain RN4220. In contrast, there was a significant accumulation of oleoyl-CoA detected in extracts from strain SA178R1/pJP106 expressing FadD. These data ruled

out the presence of an unknown protein that converts exogenous fatty acids to acyl-CoA in *S. aureus*, and were consistent with the conclusion that long-chain acyl-CoA has no role in *S. aureus* fatty acid metabolism.

Strains lacking *plsX* were fatty acid auxotrophs

The first experiment suggesting that *plsX* knockout strains were fatty acid auxotrophs came from the analysis of the growth of *B. subtilis* strain LP39 in the presence and absence of fatty acids. This strain has a chromosomal *plsX* knockout covered by an integrated xylose-inducible *plsX* gene (Paoletti *et al.*, 2007). When deprived of the xylose inducer, growth arrest occurred when PlsX levels were depleted below the threshold required for growth (Paoletti *et al.*, 2007). We found that the supplementation of strain LP39 with a mixture of branched-chain fatty acids resulted in the continued growth of the PlsX-depleted strain (Fig. 3A). These data suggested that exogenous fatty acids were able to overcome a blockade at the PlsX step, which appeared to rule out an acyl-ACP synthetase mechanism for fatty acid incorporation into phospholipid. The *B. subtilis* genetic system was complex and did not allow for the study of strains that were completely devoid of PlsX. Therefore, we constructed *S. aureus* strain PDJ39 (*plsX*) that inactivated the *plsX* gene by the insertion of a group II intron at amino acid 122 of the PlsX sequence into the parent strain SA178R1 (Fig. 3B, *inset*). Because we anticipated the *plsX* strain to be a fatty acid auxotroph, the selection steps were performed in the presence of a fatty acid supplement consisting of a mixture of *anteiso* 15:0/17:0 fatty acids (Parsons *et al.*, 2011). Strain PDJ39 (*plsX*) was indeed a strict fatty acid auxotroph (Fig. 3B). Complementation of the fatty acid-dependent growth phenotype of *plsX* strain with plasmid pPlsX expressing the *plsX* gene showed that the deficiency in PlsX was both necessary and sufficient for the growth phenotype (Fig. 3C). AFN-1252 specifically inhibits the enoyl-ACP reductase (FabI) step in the elongation cycle of FASII (Kaplan *et al.*, 2012). Although strain PDJ39 (*plsX*) was a fatty acid auxotroph (Fig. 3B), the strain remained as sensitive to AFN-1252 as the wild-type parent strain SA178R1 (Fig. 3D). Strain PDJ38 (*accD*) cannot produce malonyl-CoA, does not carry out FASII, and *accD* strains were characterized as a fatty acid and lipoate auxotrophs (Parsons *et al.*, 2011; Parsons *et al.*, 2013). Because FASII was not operational in strain PDJ38, AFN-1252 had no effect on its growth. Thus, strain PDJ39 (*plsX*) required both endogenous fatty acid synthesis from FASII and an exogenous fatty acid supplement for growth.

Fatty acid composition and phospholipid structure in strain PDJ39 (*plsX*)

Our previous study on exogenous fatty acid metabolism in *S. aureus* showed that exogenous 18:1 9 was elongated to 20:1 11 by FASII, and both 18:1 and 20:1 were incorporated into the 1-position of PtdGro (Parsons *et al.*, 2011). These data showed that exogenous fatty acids were converted to acyl-ACP for elongation by FASII and to acyl-PO₄ for utilization by PlsY. Phosphatidylglycerol (PtdGro) was the most abundant phospholipid in *S. aureus*, and the predominant PtdGro molecular species in wild-type cells consisted of 17:0 in the 1-position and 15:0 in the 2-position (Fig. 4A). The most striking aspect PtdGro structure in *S. aureus* was that 15:0 was almost exclusively found in the 2-position of PtdGro (Parsons *et al.*, 2011), illustrating the high selectivity of PlsC for the 15-carbon acyl-ACP. In wild-type strain SA178R1, there were two major PtdGro molecular species detected in cells grown

with 18:1 9 corresponding to 18:1/15:0 and 20:1/15:0 (Fig. 4B). However, in strain PDJ39 (*plsX*) only a single prominent PtdGro molecular species was detected consisting of 18:1 paired with 15:0 (Fig. 4C). About 50% of the 18:1 was elongated by FASII before incorporation in PtdGro in the wild-type strain, but there was no elongation of 18:1 9 to 20:1 11 in *PlsX*-null cells. One aspect of these experiments was that 18:1 was normally channeled to the 1-position in wild-type cells due to the substrate specificity of *PlsC* (Parsons *et al.*, 2011); however, incorporation of 15:0 into the 1-position can occur as evidenced from the existence of the 30-carbon PtdGro molecular species in strain SA178R1 (Fig. 4A). To corroborate the conclusion that exogenous fatty acids were funneled to the 1-position in *plsX* strain, it was grown with an *anteiso* 15:0 supplement and the PtdGro composition determined by mass spectrometry. Only a single molecular species consisting 15:0/15:0 PtdGro was detected in strain PDJ39 (*plsX*) confirming exogenous fatty acids were channeled into the 1-position of the phospholipids and were not elongated (Fig. 4D). These data rule out the existence of an acyl-ACP synthetase-dependent pathway for fatty acid metabolism and suggested the existence of a fatty acid kinase that converted fatty acids to acyl-PO₄, which were subsequently either converted to acyl-ACP by *PlsX* for elongation by FASII or used for acylation of the 1-position of G3P by *PlsY*.

The channeling of exogenous fatty acids to the 1-position and endogenously synthesized fatty acids to the 2-position was corroborated with metabolic labeling experiments followed by the digestion of the labeled PtdGro with phospholipase A₂ to determine the positional distribution of the label (Fig. 5). Strains SA178R1 and PDJ39 (*plsX*) were labeled with [¹⁴C]acetate and the [¹⁴C]PtdGro isolated by thin-layer chromatography. As expected, the acetate label was distributed between the 1- and 2-positions in the wild-type strain SA178R1 (Fig. 5A). However, [¹⁴C]acetate was only incorporated into the 2-position of the PtdGro isolated from the *plsX* strain (Fig. 5B). These data show that FASII only provided acyl chains for the 2-position acyltransferase (*PlsC*) in the *PlsX*-null strain. Taken together, the data in this section showed that *plsX* cells required exogenous fatty acids to acylate the 1-position of G3P and FASII to acylate the 2-position.

Analysis of FASII activity in *PlsX*-null strains

We next examined FASII activity in strain PDJ39 (*plsX*) following the removal of the exogenous fatty acid supplement. The removal of the required fatty acid supplement from the *plsX* strain in early logarithmic growth led to the cessation of growth (Fig. 6A). Initially, there was little difference between the growth of the supplemented and non-supplemented strains, but within an hour there was no increase in the optical density of the culture without fatty acid. Exogenous fatty acids were required for *de novo* fatty acid synthesis in strain PDJ39 (*plsX*). The *plsX* strain was grown to early log phase in the presence of fatty acids, the cells harvested, the fatty acid supplement removed and the cells resuspended in medium either with or without fatty acid as illustrated in Fig. 6A. The cultures were then labeled with [¹⁴C]acetate and the incorporation of label into lipids monitored for 20 min (Fig. 6B). The incorporation of acetate into lipids was reduced >98% in the absence of exogenous fatty acids showing that endogenous FASII was tightly coupled to the acquisition of extracellular fatty acids in the *PlsX*-null strain. Samples from the *plsX* strain grown in the presence of fatty acid and 30-min after the removal of fatty acids from

the medium were isolated and the ACP pool composition was determined by conformationally-sensitive gel electrophoresis followed by visualization of the ACP species by immunoblotting with anti-ACP antibodies (Fig. 6C). Normally, the ACP pool in *S. aureus* consisted primarily of non-esterified ACP (Parsons *et al.*, 2011), and only non-esterified ACP was detected in the *plsX* samples grown with fatty acids. Following the removal of the fatty acid supplement, long-chain acyl-ACP accumulated in the *plsX* strain (Fig. 6C). These data were consistent with the model that acyl-ACP utilization depended on the formation of acyl-G3P by the PlsY reaction. However, not all of the ACP was converted to acyl-ACP showing that FASII ceased before all of the ACP was depleted. This was in contrast to the conversion of all the ACP to short-chain acyl-ACP intermediates in *S. aureus* treated with the FabI inhibitor AFN-1252 (Parsons *et al.*, 2011). The accumulation of long-chain acyl-ACP correlated with the cessation of FASII, suggesting that acyl-ACP may play a regulatory role in *S. aureus* similar to the acyl-ACP regulation of FabH and acetyl-CoA carboxylase in *E. coli* (Heath and Rock, 1996; Davis and Cronan, Jr., 2001).

Metabolism of fatty acids in cell extracts

Previously, we reported that extracts from *S. aureus* formed acyl-ACP that was identified by gel electrophoresis from fatty acids, ATP and Mg^{2+} (Parsons *et al.*, 2011). These data indicated an acyl-ACP synthetase may be present; however, the analysis of PlsX-null cell extracts revealed that acyl-ACP formation required the presence of PlsX (Fig. 7A). The location of acyl-ACP and acyl- PO_4 in the thin-layer chromatography analysis was determined by preparing [^{14}C]oleoyl-ACP and partially converting it to [^{14}C]acyl- PO_4 using purified PlsX. Extracts from strain PDJ39 (*plsX*) did not produce [^{14}C]acyl-ACP from [^{14}C]oleate, ACP, ATP and Mg^{2+} , but rather the fatty acid was converted to a product that co-migrated with the acyl- PO_4 standard. The addition of purified PlsX to the (*plsX*) extract resulted in the formation of [^{14}C]acyl-ACP, and [^{14}C]acyl- PO_4 was not detected. Thus, acyl-ACP was not formed by the direct acylation of ACP by a synthetase as described in other systems (Jiang *et al.*, 2006; Jiang *et al.*, 2010), but rather through the initial formation of acyl- PO_4 followed by its transacylation to ACP via PlsX. These results showed the presence of a fatty acid kinase activity in the *S. aureus* cell extracts. Also, the experiments showed that acyl- PO_4 was rapidly converted to acyl-ACP by PlsX accounting for primarily detecting acyl-ACP in similar reactions performed previously with wild-type cell extracts (Parsons *et al.*, 2011). A filter disc assay was developed to measure fatty acid kinase activity, which was linear with the protein concentration in the PlsX-null extract (Fig. 7B). Fatty acid kinase activity required ATP plus Mg^{2+} . In addition the reaction was significantly stimulated by presenting the fatty acid substrate in Triton X-100 micelles (Fig. 7C), as are many other lipid-utilizing enzymes (Carman *et al.*, 1995). There is a single acetate kinase (*ackA*) homolog in *S. aureus*. *S. aureus* strain KB8000 (*ackA*) (Sadykov *et al.*, 2013) was not defective in [^{14}C]oleate uptake (not shown), ruling out a dual function for acetate kinase. These data detected the presence of a fatty acid kinase activity in *S. aureus* that was responsible for the formation of acyl- PO_4 from exogenous fatty acids in PlsX-null cells.

Discussion

This work reveals the existence of a fatty acid kinase pathway for the incorporation of exogenous fatty acids into the phospholipids of *S. aureus* (Fig. 1). The key intermediate in phospholipid synthesis, PtdOH, is formed by the acylation of the 1-position by PlsY followed by the acyl-ACP-dependent acylation of the 2-position by PlsC. Exogenous fatty acids access the acyltransferase system by first translocating across the bacterial cell membrane. Protonated fatty acids are known to rapidly and spontaneously flip across a phospholipid bilayer (Garlid *et al.*, 1996). This property provides a plausible mechanism for fatty acid entry into cells because there is no evidence for a protein-mediated cytoplasmic membrane transporter in any bacteria. Once the fatty acids are accessible to the cytosol, they are converted to acyl-PO₄ by an ATP/Mg²⁺-dependent fatty acid kinase (Fak). The acyl-PO₄ may then be used for the acylation of the 1-position of G3P by PlsY, or converted to acyl-ACP by PlsX. The acyl-ACP may either be elongated by FASII or utilized to acylate the 2-position via PlsC. *S. aureus* PlsC has a high selectivity for 15-carbon branched-chain fatty acids which means that exogenous fatty acids with chain lengths greater than 15 carbons are almost exclusively found in the 1-position of PtdGro. The genetic inactivation of PlsX prevents the inter-conversion of acyl-PO₄ and acyl-ACP. Thus, PlsX-null strains are not able to elongate extracellular fatty acids and require both a fatty acid supplement to supply acyl-PO₄ via fatty acid kinase (Fak) to PlsY and *de novo* FASII to supply the acyl-ACP to PlsC. The next important step in this area of research will be the identification of the gene(s) encoding the fatty acid kinase catalytic activity.

The fatty acid kinase pathway represents a new paradigm for the assimilation of extracellular fatty acids in bacteria, and stands in contrast to the pathway for exogenous fatty acid metabolism in Gram-negative bacteria. *E. coli* has been the classical model for extracellular fatty acid metabolism, and other Gram-negative bacteria possess a similar set of genes required for the activation of fatty acids derived from the environment to acyl-CoAs by acyl-CoA synthetase (FadD). Also, *E. coli* uses exogenous fatty acids as a carbon source via β -oxidation (Black and DiRusso, 1994). Many Gram-negative bacteria use fatty acids as a carbon source, and the ligation of exogenous fatty acids to CoA, rather than ACP, prevents β -oxidation intermediates from becoming intermingled with FASII intermediates. Our experiments rule out a role for acyl-CoA in *S. aureus* fatty acid metabolism (Fig. 2). This finding is consistent with the important differences in the acyl donor specificities between the *E. coli* PlsB/PlsC acyltransferase system and the PlsX/PlsY/PlsC pathway. *E. coli* PlsB and PlsC utilize either acyl-ACP or acyl-CoA, whereas *S. aureus* PlsY uses only acyl-PO₄ and PlsC uses only acyl-ACP. Some bacteria possess an acyl-ACP synthetase that belongs to the same AMP-binding protein superfamily as acyl-CoA synthetase (Jiang *et al.*, 2006); however, our experiments with PlsX-null strains rule out acyl-ACP synthetase involvement in phospholipid synthesis from extracellular fatty acids in *S. aureus*. How wide-spread the fatty acid kinase pathway for exogenous fatty acid metabolism is remains to be determined, but it seems likely that it would be present in those Gram-positive pathogens that utilize the PlsX/PlsY/PlsC pathway for phospholipid synthesis, and are able to scavenge fatty acids from the environment.

Experimental procedures

Strains, plasmids and materials

[illegible]

[1-¹⁴C]Acetate incorporation in fatty acid-treated cells

S. aureus strain RN4220 was cultured in LB medium containing 0.1% Brij-58 until A₆₀₀ = 0.5 when the culture was divided into 10 ml aliquots. Indicated amounts of fatty acid in a volume of 10 µl DMSO were added to the cultures and then were incubated at 37°C with vigorous shaking. After 30 minutes, 50 µCi of [1-¹⁴C]acetate was added to each culture which were returned to the 37°C incubator for 30 minutes. [1-¹⁴C]Acetate incorporation in DMSO-treated cells represented 100% incorporation. The cells were harvested by centrifugation, washed two times with PBS. The cell pellet was suspended in 100 µl water and lipids extracted with 360 µl chloroform:methanol:hydrochloric acid (1:2:0.02). Phases were separated after addition of 120 µl chloroform and 120 µl 2 M potassium chloride. Radiolabelled lipids were quantified by scintillation counting.

Minimal inhibitory concentration

The minimal inhibitory concentrations (MICs) for AFN-1252 against strains SA178R1, PDJ38 and PDJ39 were determined using a broth microdilution method in LB medium supplemented with 10 mg/ml bovine serum albumin and 1 mM mixture of 15:0/17:0 *anteiso* branched-chain fatty acids. *S. aureus* cultures were grown to A₆₀₀ = 1.0 and diluted 30,000-fold in medium. A 10 µl aliquot of diluted cells was added to each well of a U-bottomed, 96-well plate containing 100 µl of medium with indicated AFN-1252 concentration. A fatty acid concentration of 1 mM was used because this concentration supported normal growth of *S. aureus* fatty acid auxotrophs (*accD*) (Parsons *et al.*, 2013). The plate was incubated at 37°C for 20 h and read using a Fusion plate reader at 600 nm.

Lipodomics

PtdGro molecular species were obtained from lipid extracts from 50 ml cultures of strains SA178R1 and PDJ39 (*plsX*) grown in LB plus 10 mg/ml bovine serum albumin with or without 1 mM 18:1 or 0.2 mM *anteiso* 15:0 supplements. Lipid extraction and mass spectrometry was performed as described previously (Parsons *et al.*, 2011). Fatty acid methyl esters prepared and quantified using a Hewlett-Packard 5890 gas chromatograph as described previously (Zhang *et al.*, 2002).

The positional distribution of labeled precursors was determined by labeling strains in 10 ml cultures grown in LB medium plus 10 mg/ml bovine serum albumin containing either 5 µCi/ml [1-¹⁴C] acetate or 0.5 µCi/ml [1-¹⁴C]oleic acid and grown to A₆₀₀ = 2.0. Cells were pelleted, washed 2 × 10 ml LB containing 10 mg/ml bovine serum albumin and 2 × 10 ml H₂O. The cell pellet was suspended in 100 µl water and lipids extracted with 360 µl chloroform:methanol:hydrochloric acid (1:2:0.02). Phases were separated after addition of 120 µl chloroform and 120 µl 2 M potassium chloride. [¹⁴C]PtdGro was isolated by preparative thin-layer chromatography on Silica Gel H layers with chloroform:methanol:acetic acid (55/20/5, v/v/v). The [¹⁴C]PtdGro was digested with *Naja naja* snake venom phospholipase A₂ (Parsons *et al.*, 2011). After 3 hours, the lipids were extracted and applied to a Silica Gel H layer developed in chloroform:methanol:acetic acid (55/20/5, v/v/v) and the plate imaged with a Bioscan Imaging detector to determine the extent of digestion and the distribution of label between the 1- and 2-positions.

ACP immunoblotting

A 40 ml culture of *S. aureus* was grown to an $A_{600} = 0.7$, the cells were harvested by centrifugation and washed twice with LB medium containing 10 mg/ml bovine serum albumin. The cell pellet was split between medium either with or without oleate and grown for an additional 30 min. Cells from 20 ml cultures were centrifuged at $4000 \times g$, for 10 minutes and pellets resuspended in 110 μ l phosphate-buffered saline containing 1 mg/ml lysostaphin, 0.1 mg/ml DNase I and protease inhibitor cocktail before incubation on ice for three hours. The extracts were centrifuged at $20,000 \times g$ for 15 minutes and the supernatant removed and protein quantified using Bradford assay (Bradford, 1976). Cell lysates (30 μ g) and 100 ng of 17:0-ACP were loaded onto a 2.5 M urea, 15% acrylamide conformationally sensitive gel. The separated proteins were transferred to a polyvinylidene difluoride membrane by electroblotting. The primary anti-ACP antibody was used at 1:500 dilution and secondary anti-rabbit IgG conjugated with alkaline phosphatase at a 1:5,000 dilution. The blot was developed using the ECF substrate and the fluorescent signal recorded using a Typhoon PhosphoImager 9500.

PlsX purification

Plasmid pJY010 expressing His-tagged PlsX was transformed into BL21(DE3) Tuner cells and used to inoculate a 1 L culture of LB which was grown at 37 °C with vigorous shaking. The culture was grown until $A_{600} = 0.7$ when protein expression was induced by the addition of 1 mM IPTG. After 3 hours, the cells were harvested by centrifugation and resuspended in binding buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl). The cells were lysed using a microfluidizer and debris removed by centrifugation at $36,000 \times g$ for 15 minutes. The supernatant was applied to a Ni-NTA column and washed with increasing concentrations of imidazole. Purified PlsX was eluted in 400 mM imidazole and was subsequently dialyzed overnight against 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 100 mM NaCl and 1 mM DTT (Lu *et al.*, 2006).

Fatty acid kinase biochemical assay

Extracts for measuring fatty acid kinase activity were generated from 10 ml cultures of strain PDJ39 (*plsX*) grown in LB/BSA with 1 mM oleic acid until $A_{600} = 1.0$. Cells were centrifuged and resuspended in 110 μ l phosphate-buffer saline containing 1 mg/ml lysostaphin, 0.1 mg/ml DNase I and protease inhibitor cocktail before incubation on ice for three hours. The extracts were centrifuged at $20,000 \times g$ for 15 minutes and the supernatant removed. The radiochemical assay contained 0.1 M Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 10 mM ATP, 1% Triton X-100, 0.1 μ Ci [$1-^{14}C$] oleic acid and indicated amount of cell extract in a volume of 60 μ l. Assays were incubated at 37°C for 20 minutes before analysis. For the filter disc assay, 50 μ l of reaction mixture was spotted onto a DE81 filter disc and subsequently washed 3-times for 30 minutes with 1% acetic acid in ethanol. The discs were dried and analyzed by scintillation counting. Thin-layer chromatography was performed using 20 μ l of reaction mixture spotted onto a Silica Gel G layer, developed in chloroform:methanol:acetic acid (90/10/10) and analyzed with a Bioscan Imaging detector. The counting efficiency of the Bioscan with our Silica Gel G plates was 2%. [$1-^{14}C$]18:1-ACP was generated using [$1-^{14}C$]oleic acid, *S. aureus* ACP and the *Vibrio harveyi* acyl-

ACP synthetase enzyme (Fice *et al.*, 1993). [1-¹⁴C]oleoyl-phosphate standard was generated using [1-¹⁴C]18:1-ACP and PlsX as described previously (Lu *et al.*, 2006).

Acknowledgments

We thank Nachum Kaplan for his gift of AFN-1252 and Kenneth Bayles for strain KB8000. This research was supported by National Institutes of Health Grant GM034496 (C.O.R.), Cancer Center Support Grant CA21765 and the American Lebanese Syrian Associated Charities. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

References

- Black PN, DiRusso CC. Molecular and biochemical analyses of fatty acid transport, metabolism, and gene regulation in *Escherichia coli*. *Biochim Biophys Acta*. 1994; 1210:123–145. [PubMed: 8280762]
- Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]
- Carman GM, Deems RA, Dennis EA. Lipid signaling enzymes and surface dilution kinetics. *J Biol Chem*. 1995; 270:18711–18714. [PubMed: 7642515]
- Coleman J. Characterization of the *Escherichia coli* gene for 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (*plsC*). *Mol Gen Genet*. 1992; 232:295–303. [PubMed: 1557036]
- Cronan, JE., Jr; Rock, CO. Biosynthesis of membrane lipids. In: Neidhardt, FC.; Curtis, R.; Gross, CA.; Ingraham, JL.; Lin, ECC.; Low, KB., et al., editors. *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. Washington, D.C.: American Society for Microbiology; 1996. p. 612-636.
- D'Elia MA, Pereira MP, Chung YS, Zhao W, Chau A, Kenney TJ, et al. Lesions in teichoic acid biosynthesis in *Staphylococcus aureus* lead to a lethal gain of function in the otherwise dispensable pathway. *J Bacteriol*. 2006; 188:4183–4189. [PubMed: 16740924]
- Davis MS, Cronan JE Jr. Inhibition of *Escherichia coli* acetyl coenzyme A carboxylase by acyl-acyl carrier protein. *J Bacteriol*. 2001; 183:1499–1503. [PubMed: 11157970]
- DiRusso CC, Black PN, Weimar JD. Molecular inroads into the regulation and metabolism of fatty acids, lessons from bacteria. *Prog Lipid Res*. 1999; 38:129–197. [PubMed: 10396600]
- Fice D, Shen Z, Byers DM. Purification and characterization of fatty acyl-acyl carrier protein synthetase from *Vibrio harveyi*. *J Bacteriol*. 1993; 175:1865–1870. [PubMed: 8384617]
- Garlid KD, Orosz DE, Modriansky M, Vassanelli S, Jezek P. On the mechanism of fatty acid-induced proton transport by mitochondrial uncoupling protein. *J Biol Chem*. 1996; 271:2615–2620. [PubMed: 8576230]
- Green PR, Merrill AH Jr, Bell RM. Membrane phospholipid synthesis in *Escherichia coli*: Purification, reconstitution, and characterization of *sn*-glycerol-3-phosphate acyltransferase. *J Biol Chem*. 1981; 256:11151–11159. [PubMed: 7026564]
- Heath RJ, Jackowski S, Rock CO. Guanosine tetraphosphate inhibition of fatty acid and phospholipid synthesis in *Escherichia coli* is relieved by overexpression of glycerol-3-phosphate acyltransferase (*plsB*). *J Biol Chem*. 1994; 269:26584–26590. [PubMed: 7929384]
- Heath RJ, Rock CO. Inhibition of β -ketoacyl-acyl carrier protein synthase III (FabH) by acyl-acyl carrier protein in *Escherichia coli*. *J Biol Chem*. 1996; 271:10996–11000. [PubMed: 8631920]
- Jackowski S, Rock CO. Ratio of active to inactive forms of acyl carrier protein in *Escherichia coli*. *J Biol Chem*. 1983; 258:15186–15191. [PubMed: 6317688]
- Jiang Y, Chan CH, Cronan JE. The soluble acyl-acyl carrier protein synthetase of *Vibrio harveyi* B392 is a member of the medium chain acyl-CoA synthetase family. *Biochemistry*. 2006; 45:10008–10019. [PubMed: 16906759]
- Jiang Y, Morgan-Kiss RM, Campbell JW, Chan CH, Cronan JE. Expression of *Vibrio harveyi* acyl-ACP synthetase allows efficient entry of exogenous fatty acids into the *Escherichia coli* fatty acid and lipid A synthetic pathways. *Biochemistry*. 2010; 49:718–726. [PubMed: 20028080]

- Kaplan N, Albert M, Awrey D, Bardouniotis E, Berman J, Clarke T, et al. Mode of action, *in vitro* activity, and *in vivo* efficacy of AFN-1252, a selective antistaphylococcal FabI inhibitor. *Antimicrob Agents Chemother*. 2012; 56:5865–5874. [PubMed: 22948878]
- Kreiswirth BN, Lofdahl S, Betley MJ, O'Reilly M, Schlievert PM, Bergdoll MS, Novick RP. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* (London). 1983; 305:709–712. [PubMed: 6226876]
- Lightner VA, Larson TJ, Tailleux P, Kantor GD, Raetz CRH, Bell RM, Modrich P. Membrane phospholipid synthesis in *Escherichia coli*: Cloning of a structural gene (*plsB*) of the *sn*-glycerol-3-phosphate acyltransferase. *J Biol Chem*. 1980; 255:9413–9420. [PubMed: 6251087]
- Lu Y-J, Zhang F, Grimes KD, Lee RE, Rock CO. Topology and active site of PlsY: the bacterial acylphosphate:glycerol-3-phosphate acyltransferase. *J Biol Chem*. 2007; 282:11339–11346. [PubMed: 17308305]
- Lu Y-J, Zhang Y-M, Grimes KD, Qi J, Lee RE, Rock CO. Acyl-phosphates initiate membrane phospholipid synthesis in gram-positive pathogens. *Molec Cell*. 2006; 23:765–772. [PubMed: 16949372]
- Paoletti L, Lu Y-J, Schujman GE, de Mendoza D, Rock CO. Coupling of fatty acid and phospholipid synthesis in *Bacillus subtilis*. *J Bacteriol*. 2007; 189:5816–5824. [PubMed: 17557823]
- Parsons JB, Frank MW, Rosch JW, Rock CO. *Staphylococcus aureus* fatty acid auxotrophs do not proliferate in mice. *Antimicrob Agents Chemother*. 2013; 57:5729–5732. [PubMed: 23979734]
- Parsons JB, Frank MW, Subramanian C, Saenkhom P, Rock CO. Metabolic basis for the differential susceptibility of Gram-positive pathogens to fatty acid synthesis inhibitors. *Proc Natl Acad Sci U S A*. 2011; 108:15378–15383. [PubMed: 21876172]
- Rock CO, Goelz SE, Cronan JE Jr. Phospholipid synthesis in *Escherichia coli*. Characteristics of fatty acid transfer from acyl-acyl carrier protein to *sn*-glycerol-3-phosphate. *J Biol Chem*. 1981a; 256:736–742. [PubMed: 7005223]
- Rock CO, Goelz SE, Cronan JE Jr. Phospholipid synthesis in *Escherichia coli*. Characteristics of fatty acid transfer from acyl-acyl carrier protein to *sn*-glycerol-3-phosphate. *J Biol Chem*. 1981b; 256:736–742. [PubMed: 7005223]
- Sadykov MR, Thomas VC, Marshall DD, Wenstrom CJ, Moormeier DE, Widhelm TJ, et al. Inactivation of the Pta-AckA pathway causes cell death in *Staphylococcus aureus*. *J Bacteriol*. 2013; 195:3035–3044. [PubMed: 23625849]
- Yao J, Rock CO. Phosphatidic acid synthesis in bacteria. *Biochim Biophys Acta*. 2013; 1831:495–502. [PubMed: 22981714]
- Zhang Y-M, Marrakchi H, Rock CO. The FabR (YijC) transcription factor regulates unsaturated fatty acid biosynthesis in *Escherichia coli*. *J Biol Chem*. 2002; 277:15558–15565. [PubMed: 11859088]
- Zhong J, Karberg M, Lambowitz AM. Targeted and random bacterial gene disruption using a group II intron (targetron) vector containing a retrotransposition-activated selectable marker. *Nucleic Acids Res*. 2003; 31:1656–1664. [PubMed: 12626707]

Fig. 1.

Fatty acid metabolism in *S. aureus*.

Phosphatidic acid (PtdOH) is synthesized by the stepwise acylation of *sn*-glycerol-3-phosphate by PlsY that transfers a fatty acid to the 1-position from acyl-phosphate (acyl-PO₄) followed by PlsC that acylates the 2-position using acyl-ACP. Acyl-ACP is produced by the type II fatty acid biosynthetic pathway (FASII), and PlsX catalyzes the inter-conversion of acyl-ACP and acyl-PO₄. Exogenous fatty acids enter the cell and may be activated by either a fatty acid kinase or acyl-ACP synthetase pathway. Our experiments show strain PDJ39 (*plsX*) requires both exogenous fatty acids and *de novo* fatty acid synthesis for growth and has fatty acid kinase activity in cell extracts. These data rule out the presence of an acyl-ACP synthetase (Aas, red) and show the presence of a fatty acid kinase (Fak, green) pathway for the incorporation of exogenous fatty acids into phospholipids. The acyl-PO₄ may either be used by PlsY or converted by PlsX to acyl-ACP that can either be elongated or utilized by PlsC. The gene(s) encoding Fak are unknown.

Fig. 2.

Long-chain acyl-CoA does not have a role in *S. aureus* fatty acid uptake.

A) Inhibition of [^{14}C]acetate incorporation into fatty acid in the presence of exogenous fatty acids. Wild-type strain RN4220 was grown to an $A_{600} = 0.5$. Each fatty acid was added to LB medium containing 0.1% Brij-58 to a final concentration of 100 μM , and 30-min later [^{14}C]acetate was added. At the end of a 30 min labeling period, the cells were harvested, extracted and the amount of radioactivity in the lipid fraction determined. The data were normalized to the radiolabel in the untreated, wild-type strain set to 100% (140,000 dpm per 7×10^8 cells). Fatty acids are abbreviated as number of carbon atoms:number of double bonds. The “a” designation refers to an *anteiso* branched-chain fatty acid.

B) Expression of acyl-CoA synthetase impairs *S. aureus* growth. The *E. coli fadD* gene was cloned into the regulated expression plasmid pG164 (empty vector control) to generate pFadD, and transformed into wild-type strain SA178R1. Strains were grown

in the presence of 1 mM IPTG to induce *fadD* expression, and 18:1 was added at a concentration of 200 μ M in early log phase as indicated by the arrow.

C) Mass spectrometry analysis of the CoA thioester pool in 18:1 γ -treated cells. Strain SA178R1 harbored either pG164, the empty control expression plasmid, or pFadD, a pG164 derivative expressing *E. coli* acyl-CoA synthetase (FadD). Both strains were grown in the presence of 1 mM IPTG either in the presence or absence of 0.2 mM oleate. At an A_{600} of 1.0, the cells were harvested and processed for mass spectrometry as described (Parsons *et al.*, 2011). Propionyl-CoA was added as an internal standard and the signals for CoA, acetyl-CoA and oleoyl-CoA were normalized to the propionyl-CoA signal.

Fig. 3.

Growth phenotypes of PlsX-null strains.

A) *B. subtilis* strain LP39 was constructed to allow conditional expression of PlsX from a xylose-dependent promoter (Paoletti *et al.*, 2007). In the absence of xylose, growth arrest occurs, which was attenuated by the addition of exogenous fatty acids (FA; a 1 mM mixture of *anteiso* branched-chain 15:0/17:0 in 10 mg/ml fatty acid free bovine serum albumin plus lipoate (0.1 μ g/ml)).

B) *S. aureus* strain PDJ39 (*plsX*), a derivative of the wild-type strain SA178R1, was a fatty acid auxotroph. The strains were grown in LB medium plus 10 mg/ml fatty acid free bovine serum albumin either with or without a 1 mM oleate supplement. The inset shows a multiplex PCR genotyping result for the disrupted *plsX* gene in the *plsX* strain compared to the wild-type gene in strain SA178R1. The wild-type allele gives a 509 bp band and the mutant *plsX* allele gives a 305 bp product.

C) The strain PDJ39 (*plsX*) growth phenotype was complemented by the presence of pPlsX, a plasmid derived from pG164 that expresses the *plsX* gene. The control plasmid was the empty pG164, and both strains were grown without fatty acids.

D) Minimum inhibitory concentration for AFN-1252 in strains SA178R1 (wild-type), PDJ39 (*plsX*), and PDJ38 (*accD*). AFN-1252 is a potent inhibitor of *S. aureus* enoyl-ACP reductase and effectively blocks *de novo* fatty acid synthesis (Kaplan *et al.*, 2012). All strains were grown on LB medium with *S. aureus* branched-chain fatty acids (1 mM of a mixture of *anteiso* 15:0/17:0 in 10 mg/ml fatty acid free bovine serum albumin). Control strain PDJ38(*accD*) was a fatty acid auxotroph that cannot make any endogenous fatty acids, depends on exogenous fatty acids for acyl-PO₄ and acyl-ACP formation, and was completely refractory to AFN-1252 growth inhibition (Parsons *et al.*, 2011; Parsons *et al.*, 2013).

Fig. 4.

Phosphatidylglycerol (PtdGro) molecular species synthesized from exogenous fatty acids in wild-type strain SA178R1 and its *plsX* derivative, strain PDJ39 ($\Delta plsX$).

A) PtdGro molecular species synthesized by wild-type *S. aureus* strain SA178R1 grown in LB plus 10 mg/ml fatty acid free bovine serum medium without a fatty acid supplement. The species are labeled with the 1-position acyl chain over the 2-position acyl chain. The most abundant PtdGro molecular species consisted of 17:0 in the 1-position and 15:0 in the 2-position. These identifications were based on a more detailed analysis of PtdGro structure (Parsons *et al.*, 2011), which showed that 15-carbon fatty acids were almost exclusively localized to the 2-position of *S. aureus* PtdGro.

B) Wild-type strain SA178R1 both incorporated 18:1 ω 9 into phospholipid and elongated it to 20:1 ω 11. This resulted in two prominent molecular species containing an unsaturated fatty acid (either 18:1 or 20:1) derived from the medium and a 15-carbon branched-chain fatty acid from *de novo* biosynthesis.

- C) Strain PDJ39 (*plsX*) did not elongate 18:1 9 to 20:1 11 resulting in a single molecular species of PtdGro consisting of 18:1 and a 15-carbon branched-chain fatty acid.
- D) PtdGro molecular species derived from strain PDJ39 (*plsX*) grown in the presence of *anteiso* 15:0 (0.25 mM in 10 mg/ml bovine serum medium). Only a single PtdGro molecular species was detected consisting of two 15:0 fatty acids.

Fig. 5.

Positional distribution of labeled precursors incorporated into PtdGro.

Strain PDJ39 (*plsX*) was grown in the presence of 0.5 mM oleate, 10 mg/ml BSA. At an $A_{600} = 0.05$, 5 $\mu\text{Ci/ml}$ of [^{14}C]acetate was added and the cells grown for 7 hours. Strain SA178R1 was grown and labeled in the same manner using media without a fatty acid supplement. The lipids were extracted, [^{14}C]PtdGro was isolated by thin-layer chromatography, and digested with phospholipase A_2 . The samples were then separated by thin-layer chromatography, and the distributions of label between the 1- and 2-positions was indicated by the ratio of labeled LysoPtdGro (1-position) to FA (2-position) using the Bioscan Imaging Detector.

A) Positional distribution of [^{14}C]acetate-labeled PtdGro derived from wild-type strain SA178R1. The incomplete digestion shows the location of the substrate (PtdGro) and the two products, 1-acyl-glycerophosphoglycerol (LysoPtdGro) and fatty acid (FA).

B) Positional distribution of [^{14}C]acetate-labeled PtdGro derived from strain PDJ39 (*plsX*).

Fig. 6.

Coupling of fatty acid and phospholipid synthesis in strain PDJ39 (*plsX*).

A) Growth of strain PDJ39 (*plsX*) halts abruptly following removal of the required fatty acid supplement. The strain was grown to an A₆₀₀ of 0.5 in LB medium containing 1 mM oleate plus 10 mg/ml bovine serum albumin. The culture was then split and the oleate supplement removed by centrifugation and the cells re-suspended in media either with or without the fatty acid supplement and growth monitored.

B) Fatty acid synthesis requires exogenous fatty acids in strain PDJ39 (*plsX*). The *plsX* strain was grown to an A₆₀₀ of 0.5, and the oleate supplement removed. The cells were resuspended in media either with or without the oleate supplement and 30 min later, 5 μCi/ml of [¹⁴C]acetate was added to both cultures. At the indicated times, the cells were harvested and the amount of label incorporated into the lipid fraction was determined.

C) A representative example of the ACP pool composition under different growth conditions determined by conformationally-sensitive gel electrophoresis and immunoblotting with anti-ACP antibody. Lane 1, strain PDJ39 (*plsX*) following the removal

of the exogenous 18:1 ω -9 supplement for 30 min; Lane 2, strain PDJ39 grown with the oleate supplement; and Lane 3, *anteiso*-17:0-ACP standard.

Fig. 7.

Acyl-PO₄ and acyl-ACP synthesis in cell-free extracts.

A) Thin-layer chromatographic analysis of the reaction products formed from the incubation of cell lysates prepared from strain PDJ39 (*plsX*) containing ATP, Mg²⁺, ACP, Triton X-100 and [¹⁴C]oleate as detailed in the methods. The reaction mixtures were separated on Silica Gel G layers developed with chloroform:methanol:acetic acid (90/10/10) and the distribution of radioactivity determined with a Bioscan Imaging detector. The top trace shows the conversion of [¹⁴C]oleoyl-ACP to [¹⁴C]acyl-PO₄ by purified PlsX. The middle trace is the reaction products formed by cell-free extracts of strain PDJ39 (*plsX*) spiked with purified PlsX along with ATP, Mg²⁺ and [¹⁴C]oleate. The bottom trace is the same *plsX* extract in the absence of added PlsX.

B) A filter disc assay for fatty acid kinase was performed using the cell extracts from strain PDJ39 (*plsX*) containing ATP, Mg²⁺, Triton X-100 and [¹⁴C]oleate as detailed in the methods.

C) Maximal fatty acid kinase activity required ATP, Mg^{2+} and Triton X-100. The enzyme source was the cytosol from the *plsX* strain (300 μ g protein).