Chlamydia trachomatis Scavenges Host Fatty Acids for Phospholipid Synthesis via an Acyl-Acyl Carrier Protein Synthetase*

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Background: C. trachomatis relies on its own biosynthetic pathways to produce membrane phospholipids.

Results: C. trachomatis expresses an acyl-acyl carrier protein synthetase to activate fatty acids.

Conclusion: C. trachomatis utilizes fatty acids obtained from the host to construct phospholipids.

Significance: C. trachomatis selectively scavenges host saturated fatty acids, the most energy-expensive component needed for phospholipid synthesis.

The obligate intracellular parasite Chlamydia trachomatis has a reduced genome but relies on de novo fatty acid and phospholipid biosynthesis to produce its membrane phospholipids. Lipidomic analyses showed that 8% of the phospholipid molecular species synthesized by C. trachomatis contained oleic acid, an abundant host fatty acid that cannot be made by the bacterium. Mass tracing experiments showed that isotopically labeled palmitic, myristic, and lauric acids added to the medium were incorporated into C. trachomatis-derived phospholipid molecular species. HeLa cells did not elongate lauric acid, but infected HeLa cell cultures elongated laurate to myristate and palmitate. The elongated fatty acids were incorporated exclusively into C. trachomatis-produced phospholipid molecular species. C. trachomatis has adjacent genes encoding the separate domains of the bifunctional acyl-acyl carrier protein (ACP) synthetase/2-acylglycerolphosphoethanolamine acyltransferase gene (aas) of Escherichia coli. The CT775 gene encodes an acyltransferase (LpaT) that selectively transfers fatty acids from acyl-ACP to the 1-position of 2-acylglycerophospholipids. The CT776 gene encodes an acyl-ACP synthetase (AasC) with a substrate preference for palmitic compared with oleic acid in vitro. Exogenous fatty acids were elongated and incorporated into phospholipids by Escherichia coli-expressing AasC, illustrating its function as an acyl-ACP synthetase in vivo. These data point to an AasC-dependent pathway in C. trachomatis that selectively scavenges host saturated fatty acids to be used for the de novo synthesis of its membrane constituents.

Chlamydia trachomatis is a Gram-negative, obligate intracellular bacterial parasite with a biphasic life cycle (1). The metabolically quiescent, infectious elementary body initiates the infection by attaching to the host cell. After internalization, the elementary body differentiates into the metabolically active reticulate body, which replicates inside a specialized vacuole called the chlamydial inclusion (2, 3). There is a major reorganization and recruitment of host intracellular membrane systems to construct the expanding inclusion membrane (4). Host cell lysis releases mature elementary bodies to continue the infectious cycle. C. trachomatis has a reduced genome compared with free living Gram-negative bacteria and relies on importing building blocks from the host (4). However, C. trachomatis does not have a reduced genome when viewed from the perspective of lipid metabolism (5, 6). Rather, C. trachomatis utilizes its own de novo biosynthetic pathways to produce a unique set of disaturated molecular species of phosphatidylethanolamine (PE), 2-phosphatidylglycerol, and cardiolipin from phosphatidic acid using glucose, serine, and isoleucine from the host (6). Isoleucine is the precursor used in the synthesis of anteiso15:0, a fatty acid that is localized in the 2-position of C. trachomatis-derived phospholipids (6–8). The effectiveness of fatty acid synthesis inhibitors in blocking C. trachomatis proliferation (9) underscores the dependence of C. trachomatis on de novo phospholipid biosynthesis to produce its membrane phospholipids. The analysis of the lipidome of C. trachomatis-infected HeLa cell cultures revealed that 8% of the C. trachomatis-derived PE molecular species were 18:1/15:0 (6). C. trachomatis lacks the capacity to produce unsaturated fatty acids (6). Therefore, the 18:1 must be derived from the abundant host oleic acid pool. The existence of a mechanism in C. trachomatis to scavenge host fatty acids would be clearly advantageous because more than 95% of the energy required for the synthesis of a phospholipid is spent on the production of the fatty acid constituents (10).

The C. trachomatis Pse/Pisc acyltransferases used for phosphatidic acid formation are specific for acyl-ACP (6). Therefore, the existence of the 18:1/15:0 molecular species indicates that C. trachomatis encodes an acyl-ACP synthetase to activate

2 The abbreviations used are: PE, phosphatidylethanolamine; ACP, acyl carrier protein; FASII, bacterial type II fatty acid synthesis; GPE, sn-glyceryophosphoethanolamine; Ni-NTA, nickel-nitritriacetic acid; CtACP, C. trachomatis acyl carrier protein; PC, phosphatidylcholine.

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FIGURE 1. C. trachomatis encodes separate protein homologs to the bifunctional E. coli Aas. C. trachomatis encodes homologs to the E. coli aas gene. The E. coli aas gene encodes a bifunctional protein with 2-acyl-GPE acyltransferase activity localized in the amino-terminal domain and an acyl-ACP synthetase activity in the carboxy-terminal domain. C. trachomatis encodes adjacent genes that are homologous to the two aas domains. The CT775 gene product is related to the amino-terminal E. coli Aas acyltransferase domain (residues 11–149) (21% identity; e-value, 7e^-4) and contains the acyltransferase catalytic HX%D motif. The CT776 gene product is related to the carboxy-terminal acyl-ACP synthetase domain (residues 256–709) (27% identity; e-value, 9e^-4) and contains the domain (AFD_class_1), which corresponds to the binding site for the acyl-adenylate intermediate.

host fatty acids to be used by the bacterial acyltransferases. Escherichia coli expresses a multifunctional protein (called Aas) containing an acyl-ACP synthetase fatty acid activation domain fused to a lysophospholipid acyltransferase module (Fig. 1). The gene was named for its acyl-ACP synthetase activity before the acyltransferase function was discovered (11, 12). The protein produces acyl-ACP in vitro in the presence of chaotropic salts (13, 14), but, in vivo, acyl-ACP is not released and is not available to the enzymes of bacterial fatty acid synthesis (FASII) or the acyltransferases of phosphatidic acid synthesis (15–17). In E. coli, the enzyme functions in a salvage pathway to recycle 2-acyl-GPE formed during the maturation of lipoproteins. Topologically, lipoproteins are secreted through the inner membrane, and the subsequent processing steps, proteolytic cleavage (18) (Lgt), transfer of the diacylglycerol (19) (Lsp), and N-acylation (20) (Lnt), are located on the outer aspect of the inner membrane. The 2-acyl-GPE cycle is initiated by the transfer of a fatty acid at the 1-position of PE to the amino terminus of the lipoprotein by Lnt (21). The resulting 2-acyl-GPE product is transferred to the inner aspect of the membrane by a specific lysophospholipid transporter, LpIT (22), and reacylated by the bifunctional Aas (15). Lipoproteins in Chlamydia psittaci and C. trachomatis have the same lipid modifications as observed in E. coli (23, 24), and the genes encoding the lipid modification pathway are present (lgt, CT252; lsp, CT408; lnt, CT534). The distinguishing feature of the C. trachomatis system is that the two functions are encoded by separate genes (Fig. 1). The dissociation of the two modules suggests that the acyl-ACP synthetase protein may be able to ligate fatty acids to ACP, which, unlike in E. coli, would be released and available for FASII and/or other acyltransferases. The goal of this study was to determine whether C. trachomatis incorporates host fatty acids into its phospholipids and to identify and biochemically characterize C. trachomatis acyl-ACP synthetase (AasC), the key activating step in host fatty acid utilization by C. trachomatis.

Experimental Procedures

Materials—Cell culture supplies were from Invitrogen. Delipidated FBS was from Cocalico Biologicals. Isotopic labeled fatty acids [7,7,8,8-D4]palmitic acid (D4–16:0), [methyl-D3]myristic acid (D3–14:0), and [methyl-D3]lauric acid (D3–12:0) were from Cambridge Isotope Laboratories. Radiolabeled fatty acids ([14C]16:0 and [14C]18:1) were from American Radiolabeled Chemicals. Phospholipids were from Avanti Polar Lipids.

Chlamydia Strain, Propagation, and Isotopic Labeling—C. trachomatis serovar L2 (strain 434/Bu) was propagated by standard centrifugation infection protocol in HeLa cells maintained in DMEM containing 10% fetal bovine serum (25). For D4-palmitic acid labeling experiments in DMEM containing 10% fetal bovine serum, 100 μM of D4–16:0 was added to C. trachomatis-infected HeLa cells 18 h post-infection and incubated for another 30 h. For isotopic fatty acid labeling experiments in DMEM containing 10% delipidated FBS, cells were infected with C. trachomatis in normal medium and serum. 18 h post-infection, the medium in the cell cultures were replaced with DMEM containing 10% delipidated FBS supplemented with 100 μM D4–16:0, D3–14:0, or D3–12:0. Control HeLa cells were mock-infected (infection protocol without C. trachomatis) and then treated the same as the infected cells. After incubation, the cells were scraped for harvest, washed twice with Hank’s balanced salt solution, extracted for lipids using the Bligh-Dyer method (26), and subjected to phospholipid molecular species profiling by mass spectrometry.

PE Molecular Species Analysis—Phospholipid molecular species profiling for PE was performed as described previously (6). Phospholipid molecular species fingerprints were determined using direct infusion electrospray ionization-mass spectrometry technology (27, 28). Mass spectrometry analysis was performed using a Finnigan™ TSQ® Quantum (Thermo Electron, San Jose, CA) triple quadruple mass spectrometer. The instrument was operated in positive ion mode for PE analysis. Acyl chain lengths were assigned from the mass on the basis of product scans of the particular mass peak or predications from LipidMaps for peaks identified previously. Minor isobaric species may be present at each mass, but the major molecular species represented at each of the major mass peaks are labeled in the figures.

Molecular Biology—The lpaT (CT775), aasC (CT776), and acpP (CT236) genes of C. trachomatis serovar D/UW-3/Cx were optimized for expression in E. coli through GeneArt gene synthesis technology. For the lpaT and aasC genes, an NdeI cleavage site was engineered at the 5’ end of the gene with the start codon in the NdeI site, whereas a His8 tag, stop codon, and EcoRI cleavage site were engineered sequentially at the 3’ end of the gene. Both genes were cloned into the plasmid pET21a (New England Biolabs) and pPJ131 (29) expression vectors via the NdeI and EcoRI sites. For the acpP gene, an NdeI cleavage site was engineered at the 5’ end of the acpP gene, whereas a stop codon and a EcoRI cleavage site were engineered sequentially at the 3’ end of the gene. The acpP gene was cloned into plasmid pET28b via the NdeI and EcoRI sites. The pET16b-Vhaas plasmid was a gift from Dr. John B. Cronan (30). The Vibrio harveyi aas gene (Vhaas) was moved into the pPJ131 plasmid via the NdeI and BamHI digestion sites.

Exogenous Fatty Acid Incorporation—The pPJ131-CtaasC plasmid was transformed into E. coli strain LCH30 (aas-1 fadD88) (16). Strain LCH30 was also transformed with the
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Acyl-ACP Synthetase Assay—AasC (3.75 nM) was incubated with 10 min at 37 °C with CiACP (200 μM); 100 mM Tris-HCl (pH 8.0); 5 mM ATP; 10 mM MgCl₂; 2 mM DTT; 2% Triton X-100; and 7.5, 15, 30, 60, 120, and 240 μM of [¹⁴C]palmitic acid (55 mCi/mmol) or [¹⁴C]oleic acid (56.3 mCi/mmol) in a 40-μl reaction. After incubation, 35 μl was removed and placed on a Whatman 3MM paper disc and dried to stop the reaction. The discs were then washed twice in chloroform:methanol:acetic acid (3:6:1, v/v) to remove unbound fatty acids and dried again. The [¹⁴C]acyl-ACP product was measured in a scintillation counter.

The activity of AasC with CoA instead of ACP as the acyl acceptor was determined under similar assay conditions except with 200 μM CoA instead of 200 μM CiACP. The reactions were analyzed by thin-layer chromatography using silica gel H plates developed with chloroform:methanol:acetic acid (98:2:1, v/v). Radioactivity was quantified using a Bioscan AR-2000 detector to determine the amounts of free and CoA-bound fatty acid. Membrane and soluble fractions from strain LCH30 expressing AasC were diluted 1:20 in 20 mM Tris-HCl (pH 7.5) and incubated for 10 min at room temperature and then lysed via a cell disruptor. The cell lysate was centrifuged at 20,000 × g for 1 h, and the supernatant was chromatographed over a 4-ml Ni-NTA column. The column was washed with 20 ml of resuspension buffer and 20 ml of 10% glycerol, 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 50 mM imidazole. Purified CtACP was eluted from the column using 10% glycerol, 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 50 mM imidazole. The eluted protein was then dialyzed in a buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 1 mM DTT. The His₆ tag was cleaved from CtACP by digestion with biotinylated thrombin overnight at 4 °C with gentle rotation. A small Ni-NTA column was used to remove the cleaved His₆ tag, and streptavidin-agarose was used to remove the biotinylated thrombin. The apoACP was converted to ACP by incubation in a mixture of 50 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 4 μM His₆-tagged Streptococcus pneumoniae [ACP]synthase, and 4 mM CoA to a final volume of 1 ml for 1 h at 37 °C (9). His₆-tagged synthase was removed with a Ni-NTA column. Samples were loaded into a prepacked PD-10 desalting column (GE Healthcare) pre-equilibrated with 25 ml of 20 mM Tris-HCl (pH 7.5). Purified ACP was eluted with 20 mM Tris-HCl (pH 7.5) and concentrated with a 5000 molecular weight cutoff Amicon concentrator.

The expression and purification of AasC was similar to CtACP, except BL21(DE3) Rosetta cells expressing the pET21a-aasC plasmid was used instead, and the His₆ tag was not removed. The association of AasC with membranes was determined using strain LCH30 expressing the pPJ131-CtAsC plasmid. The cells (1 liter) were grown to an A₆₀₀ of 1 at 37 °C and then harvested and resuspended in 20 ml of Tris-HCl, pH 7.5. The cells were lysed via a French press, and the cell debris was pelleted via centrifugation at 20,000 × g at 4 °C for 30 min. The supernatant was further centrifuged at 100,000 × g at 4 °C for 30 min to separate the cell membranes from the soluble fraction. The amount of protein in each fraction was determined using the Bradford assay (31). The AasC activity in the fractions was determined.

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gation at 20,000 × g at 4 °C for 30 min. The supernatant was then centrifuged at 100,000 × g at 4 °C for 30 min to separate the cell membranes from the soluble fraction. The cell membrane fraction from each 50 ml of original cell culture was resuspended in 50 μl of 100 mM Tris-HCl (pH 8.2), 150 mM NaCl, 2 mM MgCl₂, and 0.1% Brij-58 (reaction buffer) and stored for up to 2 days at 4 °C. A reaction composed of acyl-GPC, acyl-GPE, or egg acyl-GPC was blown down and resuspended in 10 μl of reaction buffer, and the purified membranes containing LpaT and 10 μl of [¹⁴C]16:0-ACP were added to a final volume of 40 μl and incubated for 30 min at 37 °C. The reaction was stopped, and the lipid fraction was extracted via the Bligh-Dyer method (26). The lipid fraction was chromatographed on silica H plates (Analtech) developed with chloroform:methanol:acetic acid:water solvent system (50:25:8:4, v/v). The chromatographic motilities were graphed on silica H plates (Analtech) developed with chloroform:methanol:acetic acid:water solvent system (50:25:8:4, v/v). The distribution of radioactivity on the thin-layer plate was quantified using a Bioscan AR-2000 detector.

Results

Incorporation of Host-derived Fatty Acids—The major C. trachomatis PE molecular species contained a 16:0 fatty acid at the 1-position and an anteiso15:0 fatty acid at the 2-position (6). The branched-chain 15:0 is made by the C. trachomatis FASII, but the 16:0 fatty acid may be derived from either the HeLa cell host or C. trachomatis. C. trachomatis synthesizes fatty acids through the FASII cycle by the two-carbon elongation of acyl-ACP, which is the preferred acyl donor for the C. trachomatis acyltransferase system (PlsE + PlsC) for phosphatidic acid synthesis (6). In contrast, the product of the host multidomain fatty acid synthase enzyme is nonesterified 16:0 fatty acid, and the host acyl-CoA synthetases convert 16:0 into 16:0-CoA. Acyl-CoA can then be elongated and desaturated or used by the host acyltransferase systems. Therefore, to determine whether C. trachomatis could incorporate exogenous host fatty acids, C. trachomatis-infected HeLa cells were labeled with the isotopic D4–16:0 fatty acid, and the PE molecular species of the infected cell system were determined by mass spectrometry (Fig. 2). Incorporation of D4–16:0 into uninfected host PE molecular species revealed the appearance of a prominent new peak at m/z = 722 that corresponded to the incorporation of D4–16:0 into the host 16:0/18:1 PE molecular species (Fig. 2C). In the infected cell population, D4–16:0 was incorporated into 16:0/18:1 host PE, and, in addition, the new peak at m/z 682 indicated the incorporation of D4–16:0 into the 16:0/15:0 PE molecular species by the C. trachomatis acyltransferases (Fig. 2D). These data indicated that C. trachomatis was able to incorporate extracellular 16:0 via the host into its de novo phospholipid biosynthetic pathway.

The next series of experiments were designed to determine whether medium-chain fatty acids would be elongated in the infected cell system. In the next series of labeling experiments, 10% delipidated serum in the medium was used to increase incorporation of the labeled fatty acid by removing the other competing fatty acid species in normal serum. The amount of D4–16:0 incorporation was increased significantly with 10% delipidated serum, allowing additional features to be detected (Fig. 3, A and B). D4–16:0 was incorporated into host 16:0/18:1 PE and was also elongated and incorporated into 18:0/18:1 molecular species (m/z 750) (Fig. 3, A and B). Labeling uninfected cells with D3–14:0 showed that 14:0 was elongated to 16:0 and 18:0 by HeLa cells and used to synthesize 16:0/18:1 and 18:0/18:1 PE molecular species (Fig. 3C). In the infected cultures, D4–14:0 was also elongated and incorporated into the C. trachomatis-derived disaturated PE molecular species 16:0/14:0 and 16:0/15:0 (Fig. 3D). These experiments could not determine whether the 14:0 was elongated by the acyl-CoA-dependent host pathway or by the acyl-ACP-dependent C. trachomatis pathway. We did not detect D3–12:0 incorporation into PE molecular species in uninfected HeLa cells (Fig. 3E). These data indicated that 12:0 was not a substrate for the host acyltransferases, and the absence of molecular species contain-
ing heavy 16:0 or 18:0 fatty acids showed that 12:0 was not elongated by HeLa cells (Fig. 3E). In contrast, D3–12:0 was elongated to 14:0 and 16:0 in the infected cell cultures, and these heavy fatty acids were primarily paired with 15:0 in bacterially derived disaturated PE molecular species (Fig. 3F). Elongated, heavy fatty acids derived from D3–12:0 were only detected in C. trachomatis-derived PE molecular species (Fig. 3F). These data showed the C. trachomatis-dependent elongation of D3–12:0 to D3–14:0 and D3–16:0 by bacterial FASII prior to incorporation into PE. These data were consistent with D3–12:0 being activated by an acyl-ACP synthetase.

These experiments definitively established that fatty acids were trafficked from the medium via the host to C. trachomatis, where they were metabolized further. However, quantification of the extent of C. trachomatis fatty acid acquisition from the host was challenging because of many unknowns in the labeling experiments. When labeled fatty acid was added to the medium, it was diluted out by an unknown amount by the unlabeled fatty acid in the serum. The fatty acid imported into the cell was then diluted by the fatty acid being produced by host fatty acid synthase, and the fatty acid imported into C. trachomatis was diluted again by the fatty acid produced by bacterial FASII. These differences mean that the fatty acid labeling of host and bacterial lipid cannot be compared directly. There were also bacterial and host phospholipids present at the beginning of the labeling period, and the contribution of this pre-existing, unlabeled phospholipid to the amounts determined in the spectra was unknown.

**Characterization of Chlamydial AasC**—C. trachomatis encodes for a homologue (CT776, aasC) to the C-terminal acyl-ACP synthetase domain of the bifunctional E. coli Aas (Fig. 1). A series of experiments was designed to verify that AasC functions as an acyl-ACP synthetase. E. coli strain LCH30 (aas-1 fadD88) was unable to incorporate any exogenous fatty acids into phospholipids because of the inactivation of both synthases capable of activating fatty acids (16). Accordingly, strain LCH30 harboring the empty expression vector was unable to incorporate any D3–14:0 into phospholipids (Fig. 4A). Strain LCH30 expressing the E. coli acyl-CoA synthetase did incorporate low levels of D3–14:0 into the phospholipids, but, because of the inability of E. coli to elongate acyl-CoA and the substrate specificities of the acyltransferases, 14:0 incorporation was limited to the minor PE molecular species containing 14:0 (Fig. 4B). A second control was the expression of V. harveyi Aas in strain

**FIGURE 2. Incorporation of exogenous D4–16:0 in uninfected and C. trachomatis-infected HeLa cells.** Mock-infected HeLa and C. trachomatis-infected HeLa cells were labeled with D4–16:0 fatty acids (100 μM) between 18–48 h post-infection. The molecular species profiles for the PE were determined by mass spectrometry as described under “Experimental Procedures.” The identities of the major molecular species are annotated in the figures. C. trachomatis-derived molecular species containing heavy fatty acids are highlighted in red, and isotopically labeled host-derived molecular species are shown in blue. The spectra shown are representative examples from two biological replicates. A, PE molecular species profile of uninfected HeLa cells. B, PE molecular species profile of C. trachomatis-infected HeLa cells. Note the small percentage of 18:1/15:0 molecular species. C, PE molecular species profile of uninfected HeLa cells labeled with D4–16:0. D, PE molecular species profile of C. trachomatis-infected HeLa cells labeled with D4–16:0.
In this strain, D3–14:0 was elongated to D3–16:0 prior to incorporation into phospholipid (Fig. 4C). These data showed that the conversion of D3–14:0 to acyl-ACP allowed the elongation of the 14-carbon fatty acid by FASII. The expression of the putative *C. trachomatis* AasC in strain LCH30 also resulted in the elongation of D3–14:0 to D3–16:0 prior to its incorporation into PE (Fig. 4D). These data were consistent with the AasC functioning as an acyl-ACP synthetase.

A His$_6$-tagged version of the *aasC* gene was expressed in BL21(DE3) cells, and AasC was purified to more than 90%
purity (Fig. 5A). Purified AasC catalyzed the ATP-dependent ligation of free fatty acids to ACP. CoA could not replace ACP as the acyl acceptor (Fig. 5B). Although the E. coli Aas is an integral membrane protein, the acyl-ACP synthetase domain does not contain the predicted transmembrane regions and is likely tethered to the membrane by the acyltransferase domain. The V. harveyi acyl-ACP synthetase is a soluble monomer (30). Given that long chain free fatty acids such as 16:0 are found in the membrane, we determined whether AasC was associated with the membrane by measuring the specific activity of AasC in the membrane and soluble fractions of strain LCH30 expressing aasC. The membrane fraction had twice the enzyme-specific activity as the soluble fraction (Fig. 5C), demonstrating that AasC was a peripheral membrane protein. This purity (Fig. 5A). Purified AasC catalyzed the ATP-dependent ligation of free fatty acids to ACP. CoA could not replace ACP as the acyl acceptor (Fig. 5B). Although the E. coli Aas is an integral membrane protein, the acyl-ACP synthetase domain does not contain the predicted transmembrane regions and is likely tethered to the membrane by the acyltransferase domain. The V. harveyi acyl-ACP synthetase is a soluble monomer (30). Given that long chain free fatty acids such as 16:0 are found in the membrane, we determined whether AasC was associated with the membrane by measuring the specific activity of AasC in the membrane and soluble fractions of strain LCH30 expressing aasC. The membrane fraction had twice the enzyme-specific activity as the soluble fraction (Fig. 5C), demonstrating that AasC was a peripheral membrane protein. This molecular species profile of strain LCH30 expressing the following vectors labeled with 100 μCi of either [1-14C]16:0 or [1-14C]18:1 for 15 min, followed by lipid extraction and the measurement of labeled phospholipid.

FIGURE 5. Biochemical characterization of AasC. A, a His_{6}-tagged version of AasC was expressed in BL21(DE3) Rosetta cells and purified to more than 90% purity by metal chelation chromatography. B, AasC-catalyzed acyl-ACP and acyl-CoA synthetase activities were compared using [1-14C]16:0 as the substrate and 200 μM ACP or CoA as the acyl acceptor. C, strain LCH30 expressing AasC was lysed, and the membrane and soluble fractions were isolated. The two fractions were assayed for acyl-ACP synthetase activity to determine the AasC-specific activity in the two fractions. D, the acyl chain specificity of AasC was determined. AasC preferred the saturated, straight-chain 16:0 fatty acid (k_{cat} 7.2 ± 0.1 s^{-1} and apparent K_{m} 35.3 ± 5.8 μM) over the unsaturated 18:1 fatty acid (k_{cat} 2.0 ± 0.3 s^{-1} and apparent K_{m} 180.4 ± 54.7 μM). E, the exogenous fatty acid incorporation into E. coli strain LCH30 expressing aasC was determined by labeling with 10 μCi of either [1-14C]16:0 or [1-14C]18:1 for 15 min, followed by lipid extraction and the measurement of labeled phospholipid.
same behavior was described for FadD (acyl-CoA synthetase) in *E. coli* (34). Like *E. coli* FadD, AasC was associated with the membrane where the fatty acid substrate was located.

Acyl chain selectivity of the AasC for 16:0 and 18:1, the two most abundant fatty acids made by the HeLa cell host, was determined using steady-state kinetics. AasC preferred the saturated, straight chain 16:0 fatty acid ($k_{cat}$ 7.2 ± 0.1 s$^{-1}$, apparent $K_m$ 35.3 ± 5.8 µM) over the unsaturated 18:1 fatty acid ($k_{cat}$ 2.0 ± 0.3 s$^{-1}$, apparent $K_m$ 180.4 ± 54.7 µM) (Fig. 5D). The preference for 16:0 over 18:1 was corroborated by comparing the labeling of strain LCH30 expressing aasC with equal concentrations of either [14C]16:0 or [14C]18:1. More than twice the amount of [14C]16:0 was incorporated compared with [14C]18:1 (Fig. 5E). This experiment indicated a preference of AasC for saturated fatty acids, but the ratio of incorporation cannot be used as a precise measure of AasC substrate selectivity because incorporation is also influenced by the substrate selectivities of the acyltransferases. The selectivity for 16:0 over 18:1 by the AasC was consistent with the observed acyl chain composition of *C. trachomatis* phospholipids, where 16:0/15:0 was the most abundant *C. trachomatis* PE species, whereas 18:1/15:0 was a minor species. Importantly, the highly related *E. coli* Aas exhibits selectivity for saturated fatty acids (13, 14), consistent with its selectivity for fatty acids that are destined to be incorporated into the 1-position of PE, which is normally occupied by 16:0. Together, these results showed that AasC functions as a bona fide acyl-ACP synthetase with a substrate selectivity that was consistent with the molecular species of PE produced by *C. trachomatis*.

**Characterization of C. trachomatis LpaT**—Bioinformatic analysis showed that *C. trachomatis* has a gene upstream of aasC that corresponded to the 2-acyl-GPE acyltransferase domain (CT775, lpaT) of *E. coli* aas (Fig. 1). The clear prediction from the gene organization and bioinformatic analysis was that lpaT was the *C. trachomatis* homolog of 2-acyl-GPE acyltransferase and would function to recycle 2-acyl-GPE arising from lipoprotein biogenesis. An abundant *C. trachomatis* lipoprotein with an acylated amino terminus is known (24), and the transferase and would function to recycle 2-acyl-GPE arising from lysophospholipid metabolism. An abundant 2-acyl-GPE acyltransferase catalyzing this last step in lipoprotein maturation (CT534, Lnt) is present in the genome, suggesting that LpaT would have the same function in *C. trachomatis* as in *E. coli*. A complementation approach was used to test this prediction in *vivo*. *E. coli* strain LCH66 (aas-1 pldA1 pldB12) accumulates 2-acyl-lysoPE arising from lipoprotein synthesis because of the inactivation of the *E. coli* 2-acyl-GPE salvage pathway (aas-1) and two phospholipases (pldA1 and pldB12) (16). Accordingly, acyl-GPE was detected by metabolic labeling of strain LCH66 (Fig. 6, lane 1). Acyl-GPE did not accumulate in strain LCH66 engineered to express lpaT (Fig. 6, lane 2). These experiments showed that lpaT expression restored the missing 2-acyl-GPE acyltransferase activity in *vivo*. As reported previously (16), control membrane fractions derived from strain LCH30 (aas-1) lacked 2-acyl-GPE acyltransferase activity in *vitro* (data not shown). However, membranes isolated from strain LCH30 expressing lpaT exhibited acyl-GPE and acyl-GPC acyltransferase activity using [14C]16:0-ACP as the acyl donor. The lack of phospholipid headgroup specificity of LpaT was a property shared with the *E. coli* acyltransferase (22). The positional distribution of the incorporated [14C]16:0 was determined by digesting the phospholipid products with snake venom phospholipase A$_2$ followed by separation of the 1- and 2-position fatty acids by thin-layer chromatography. These data showed that [14C]16:0-ACP was selectively incorporated into the 1-position of PC (Fig. 7A) or PE (Fig. 7B). We concluded that LpaT was an acyl-ACP-dependent, 1-position-selective lysophospholipid acyltransferase like its *E. coli* homolog.

This conclusion was not consistent with the recent description of LpaT as a 1-acyl-GPC acyltransferase that cooperates with a host acyl-CoA binding protein to reacylate the 2-position of host 1-acyl-GPC with a bacterially derived branched-chain fatty acid described by Soupene et al. (35). Lipidomic analyses have definitively ruled out the modification of host PC with bacterially derived branched-chain fatty acids (6). Therefore, LpaT cannot function in such a pathway. Soupene et al. (35) also did not determine the positional distribution of fatty acids in their product. LpaT activity measured with commercial egg 1-acyl-GPC used by Soupene et al. (35) showed the formation of [14C]PC from [14C]16:0-ACP (Fig. 7C). The digestion of the product with phospholipase A$_2$ (Fig. 7D) showed that the label was selectively incorporated into the 1-position (Fig. 7E). This result arises from lysophospholipids being a mixture of 1- and 2-position isomers, a situation that cannot be completely avoided because of the well-known acyl chain migration in lysophospholipids (36). This physical property of lysophospholipid...

**FIGURE 6. Complementation of the E. coli 2-acyl-GPE acyltransferase defect by lpaT.** The empty plasmid (pPJ131) and the plasmid expressing lpat (pPJ131-lpaT) were transformed into *E. coli* strain LCH66 (aas-1 pldA1 pldB12) lacking 2-acyl-GPE acyltransferase and phospholipase activities. The strains were labeled for 3 h with [32P]orthophosphate, and the composition of the lipid extract was determined by PhosphorImager analysis of a thin-layer chromatogram. As expected, strain LCH66 harboring the empty plasmid accumulated abnormally high levels of acyl-GPE (lane 1). The strain expressing lpaT prevented acyl-GPE accumulation (lane 2). The autoradiogram shown is a representative example from two biological replicates. PG, phosphatidylglycerol; CL, cardiolipin.
ids requires the further evaluation of the product structure to
determine the positional specificities of lysophospholipid acyl-
transferases. We concluded that LpaT was a 2-acyl-GPE acyl-
transferase involved in recycling 2-acyl-GPE generated as a
byproduct of lipoprotein biosynthesis.

Discussion

Our experiments lead to the model for C. trachomatis scav-
enging of host fatty acids outlined in Fig. 8. The fundamental
roles of C. trachomatis FASII in providing acyl-ACP for phos-
pholipid and lipid A biosynthesis have been established (6, 9).
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**FIGURE 8. Model for the role of AasC in C. trachomatis lipid metabolism.** Autonomous phospholipid synthesis is shown. The fatty acid synthesis module (FASII) supplies acyl-ACP to the PlsE and PlsC acyltransferases of phospholipid biosynthesis and to the lipid A acyltransferases that initiate lipo-oligosaccharide (LOS) biosynthesis, as described previously (6, 9). The new enzymes characterized in this work are shown in light blue. AasC, the C. trachomatis acyl-ACP synthase, feeds into the pathway by ligating free fatty acids acquired from the host to ACP. The resulting acyl-ACP may be used by the acyltransferases to initiate phospholipid synthesis. Alternatively, acyl-ACP may enter the fatty acid synthesis cycle via FabF, and the resulting acyl-ACP products may be distributed to the phospholipid and lipo-oligosaccharide acyltransferases. LpaT functions as the acyl-ACP-dependent acyltransferase to recycle 2-acyl-GPE arising from the Lnt reaction in lipoprotein maturation. G3P, glycerol-3-phosphate; FA, phosphatidic acid; FA, fatty acid.

Phosphatidic acid, the key intermediate in phospholipid synthesis, is produced by the sequential acylation of glycerol-3-phosphate by the PlsE and PlsC acyltransferases utilizing acyl-ACP as the acyl donor (Fig. 8). The new proteins characterized in this study fit into the metabolic scheme as salvage pathway components that recycle 2-acyl-GPE arising from C. trachomatis lipoprotein biosynthesis (LpaT) or scavenge host fatty acids (AasC) (Fig. 8). The substrate preference of AasC for palmitic acid acts as a filter that discriminates against more abundant host unsaturated fatty acids like oleate. Nonetheless, ~8% of bacterially produced PE contained oleate, but polyunsaturated fatty acids paired with 15:0 were not detected. These analytical data illustrate that the discrimination against abundant host monounsaturated fatty acids is not absolute, but the system effectively excludes host polyunsaturated fatty acids. Host saturated fatty acids may be derived from type I fatty acid biosynthesis, imported from the medium, or perhaps released from host lipids by hydrolyases. How host fatty acids are trafficked to and transported into the bacterial inclusion is an open question, but it seems likely that host factors play a key role in this process. C. trachomatis encodes several esterases of unknown function, one or more of which may be secreted and participate in the hydrolysis of host lipids to release saturated fatty acids. Fatty acids in the inclusion lumen must be transported to the inner aspect of the C. trachomatis inner membrane to make them available to ACP and AasC. Bacterial transporters that catalyze fatty acid transbilayer movement are not known, and it is thought that the fatty acids enter the cell by spontaneously flipping across the inner membrane in a process driven by the electrochemical gradient (37). The fatty acids are converted to acyl-ACP by AasC and the released acyl-ACP may either be used for phospholipid synthesis or enter FASII and be elongated (Fig. 8). AasC has retained the selectivity for saturated fatty acids exhibited by E. coli Aas (14), and it is therefore ideally suited to scavenge the type of acyl chains that are used by the PlsE acyltransferase. The elongated fatty acids may be converted to 18- and 20-carbon \( \beta \)-hydroxyacyl-ACPs, which would be used by the acyltransferases of lipo-oligosaccharide biosynthesis (CT531 = lpxA; CT243 = lpxD) to construct the unique C. trachomatis lipid A structures (38). Fatty acid synthesis is an energy-intensive process (10), and the energetic advantage of scavenging fatty acids from the host is obvious. Supplementing cells engineered to overexpress fatty acid binding protein with palmitate increased the titers of C. trachomatis (39), consistent with the energy-saving view of fatty acid scavenging. However, this process is constrained to not significantly perturb the production of predominately disaturated phospholipid molecular species C. trachomatis utilizes to construct its membrane systems.

Whether AasC activity is required for C. trachomatis development or represents a non-essential salvage pathway remains an open question. All bacterial pathogens characterized to date encode for pathways to incorporate exogenous fatty acids that spare energy for other cellular activities (37). Therefore, viewing AasC as an opportunistic pathway with a role in increasing the overall cellular yield of C. trachomatis by sparing energy for other biosynthetic pathways is consistent with the bacterial paradigm. Endogenous C. trachomatis fatty acid synthesis is essential for the replication of C. trachomatis on the basis of the blockade of growth by a FASII inhibitor (9). However, this fact alone cannot be used to conclude that C. trachomatis does not support its growth with host fatty acids. Treatment with FASII inhibitors blocks both fatty acid activation and de novo synthesis because acyl-ACP intermediates accumulate at the blocked step, depriving the system of the ACP needed to activate the incoming fatty acids (40). The idea that host straight-chain saturated fatty acids are required for C. trachomatis growth seems unlikely because the spectrum of products produced by Chlamydia is the same as those of other bacteria with the same complement of biosynthetic machinery. For example, Staphylococcus aureus synthesize both straight- and branched-chain, saturated fatty acids using the same FASII pathway components found in C. trachomatis (40). The contribution of host fatty acid synthesis to the C. trachomatis life cycle would be clearly revealed by genetically knocking out aasC and examining the impact on phospholipid structure and organism development. Such genetic manipulations in C. trachomatis are difficult, but recently a random mutagenesis library covering the whole genome has been reported for C. trachomatis (41). Although several missense mutations in the aasC gene have been reported, the mutations did not occur in residues of known function in the synthetase protein family, and it remains to be determined whether any of these mutants would be useful for interrogating the role of AasC in the C. trachomatis developmental cycle.

**Author Contributions**—J. Y. and C. O. R. designed the study and wrote the first draft. M. W. F. performed the mass spectrometry, and V. J. D. performed the AasC enzymology (Fig. 5). All authors read and approved the final version.

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