Chlamydia trachomatis Relies on Autonomous Phospholipid Synthesis for Membrane Biogenesis**

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Background: C. trachomatis has a reduced genome and was thought to obtain phospholipids as well as other nutrients from the host.
Results: The new phospholipid molecular species that appear in infected cells are produced by C. trachomatis.
Significance: C. trachomatis relies on autonomous phospholipid synthesis.

The obligate intracellular parasite Chlamydia trachomatis has a reduced genome and is thought to rely on its mammalian host cell for nutrients. Although several lines of evidence suggest C. trachomatis utilizes host phospholipids, the bacterium encodes all the genes necessary for fatty acid and phospholipid synthesis found in free living Gram-negative bacteria. Bacterially derived phospholipids significantly increased in infected HeLa cell cultures. These new phospholipids had a distinct molecular species composition consisting of saturated and branched-chain fatty acids. Biochemical analysis established the role of C. trachomatis-encoded acyltransferases in producing the new disaturated molecular species. There was no evidence for the remodeling of host phospholipids and no change in the size or molecular species composition of the phosphatidylycerine pool in infected HeLa cells. Host sphingomyelin was associated with C. trachomatis isolated by detergent extraction, but it may represent contamination with detergent-insoluble host lipids rather than being an integral bacterial membrane component. C. trachomatis assembles its membrane systems from the unique phospholipid molecular species produced by its own fatty acid and phospholipid biosynthetic machinery utilizing glucose, isoleucine, and serine.

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The abbreviations used are: EB, elementary body; ACP, acyl carrier protein; C. trachomatis, Chlamydia trachomatis; FASII, bacterial type II fatty acid synthesis; RB, reticulate body; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; SM, sphingomyelin; PG, phosphatidylglycerol; CL, cardiolipin; Gro-3-Ph, sn-glycerol-3-phosphate; D4-Etn, ethanolamine-1,1,2,2-tetra-d4-amine.

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Chlamydia trachomatis is a Gram-negative, obligate intracellular bacterial parasite with a biphasic life cycle (1). The metabolically quiescent, extracellular, and infectious elementary body (EB) initiates the infection by attaching to the host cell.

After internalization, the EB differentiates into the metabolically active, noninfectious reticulate body (RB), which replicates inside a specialized vacuole called the chlamydial inclusion (2, 3). There is a major re-organization and recruitment of host intracellular membrane systems to construct the expanding inclusion membrane (4). Host cell lysis releases mature EBs (~2 × 104 EB/cell (5)) to continue the infectious cycle. C. trachomatis has a reduced genome (~1 million bp) compared with free living Gram-negative bacteria (Escherichia coli, ~5 million bp) and relies heavily on importing building blocks from the host to assemble its unique complement of DNA, RNA, and protein (4).

C. trachomatis does not have a reduced genome when viewed from the perspective of lipid metabolism. A bioinformatic analysis reveals that C. trachomatis encodes all the genes typically found in Gram-negative bacteria (i.e. E. coli) that are necessary for type II fatty acid synthesis (FASII) and the synthesis of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) (Fig. 1) (6). C. trachomatis does not encode genes to introduce a double bond into the growing acyl chain suggesting that only saturated fatty acids are produced by the bacterial pathway. C. trachomatis encodes a branched-chain ketoacid dehydrogenase to supply the precursors for branched-chain fatty acid biosynthesis. As in free-living bacteria, FASII produces acyl-ACP for phospholipid synthesis that is initiated by the sn-glycerol-3-phosphate (Gro-3-P) acyltransferases (Fig. 1). C. trachomatis is predicted to encode a unique bacterial Gro-3-P acyltransferase with sequence similarity (38% identity) to the soluble Gro-3-P acyltransferases of plant plastids (7). This gene was designated plsE to distinguish it from the integral membrane Gro-3-P acyltransferases typically found in bacteria (plsB and plsV) (8). Phosphatidic acid (PA) produced by the acyltransferases is predicted to be converted into PE, PG, and CL. C. trachomatis also encodes the acyltransferases used to synthesize lipid A from FASII intermediates (CT531 = lpxA; CT243 = lpxD; CT010 = htrB), and it appears to have the fatty acid-activating/lysophospholipid acyltransferase components

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![Membrane Phospholipid Synthesis Diagram](image-url)

The bioinformatic model for *C. trachomatis* phospholipid synthesis. *C. trachomatis* encodes the genes for the synthesis of PE, PG, and CL that are conserved in all sequenced *C. trachomatis* serovars. The locus tags for these genes in *C. trachomatis* strain D/UW-3/Cx are annotated below the enzyme symbols. The genes were easily identified based on comparisons with well-characterized homologues in *E. coli* (6) with the exception of the Gro-3-P acyltransferase. The gene predicted to encode this activity (*plsE*) is most closely related to the soluble acyltransferase of plant plastids (7). *C. trachomatis* lacks the genes for unsaturated fatty acid biosynthesis (65), but like many bacteria, *C. trachomatis* produces branched-chain fatty acids as a substitute for unsaturated fatty acids. *C. trachomatis* has a branched-chain ketoacid dehydrogenase (*bkd*) to supply the primer for branched-chain fatty acid biosynthesis, and *gpdA* (CT714) produces the Gro-3-P for the acyltransferases. The *C. trachomatis* cls gene was related to all three *E. coli* cls genes (e-value of 1e-16 to cls1, 6e-9 to cls2, and 5e-5 to cls3). The multiple enzymes that dephosphorylate phosphatidylglycerolphosphate were difficult to identify in *E. coli* (64), and the phosphatase in *C. trachomatis* was not identified with confidence.

Although it is widely understood that *C. trachomatis* appears capable of synthesizing its own lipids, two main lines of evidence have led to the conclusion that *C. trachomatis* depends on the acquisition of host phospholipids for membrane biogenesis and proliferation (13, 14). First, isolated *C. trachomatis* have a phospholipid class composition that reflects the composition of the host cell (15–19). One distinct difference is that branched-chain fatty acids (*i.e.* anteiso-15:0; number of carbons/number of double bonds) are found in the 2-position of phospholipids in *C. trachomatis*-infected HeLa cells (20). Mammalian cells do not make this type of fatty acid, but radioactivity from the branched-chain fatty acid precursor isoleucine is incorporated into host phosphatidylcholine (PC) leading to the idea that host phospholipids were remodeled with branched-chain fatty acids by a deacylation-reacylation pathway (18). Host cPLA$_2$ is activated during infection and is proposed to initiate the remodeling pathway by hydrolyzing fatty acids from the 2-position of host phospholipids (21). Recently, a *C. trachomatis* acyltransferase was reported to reacylate lyso-PC to thereby complete the remodeling process (22). Second, *C. trachomatis* co-opts host trafficking pathways to mobilize host cell membrane lipids (PC, cholesterol, and sphingomyelin (SM)) from cellular organelles to assemble the inclusion membrane with host organelles. SM and cholesterol are trafficked from the Golgi (15, 16, 19, 23–25), and components of the host high density lipoprotein biogenesis machinery are recruited to the inclusion membrane to facilitate the translocation of PC (26). Lipid transfer is facilitated by the close association of the *C. trachomatis* inclusion with host organelles such as the endoplasmic reticulum (23, 27–30) and multivesicular bodies (31–33). *C. trachomatis* is also proposed to utilize host lipid droplets and fatty acid-binding proteins to assimilate lipids (24, 34, 35). A functioning inclusion membrane is critical to *C. trachomatis* proliferation, so it is not clear whether the lower *C. trachomatis* titer observed when lipid trafficking is inhibited arise from insufficient lipid for *C. trachomatis* and/or inclusion membrane formation. This has been a particularly difficult area for study because the inclusion membrane has not been isolated in vitro, and there are tight connections between *C. trachomatis* and the host-derived inclusion membrane through a structure recently described as the "pathogen synapse" (3, 36, 37). These tight connections suggest that it may be difficult to isolate bacteria devoid of host membrane contamination.

The goal of this study is to determine the contributions of *C. trachomatis* phospholipid synthesis and host phospholipid modification. We find that *de novo* *C. trachomatis* lipid synthesis is responsible for the increase in abundance of PE, PG, and CL in *C. trachomatis*-infected cultures. Lipidomic analyses show that there is no modification of host phospholipids with bacterially derived fatty acids and that *C. trachomatis* produces a unique constellation of disaturated phospholipid molecular.
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species that are not found in the host. These results establish that C. trachomatis uses its own biosynthetic pathways (Fig. 1) to synthesize unique molecular species of PE, PG, and CL that are essential for the biogenesis of its membrane systems.

Experimental Procedures

Materials—Unless otherwise stated, chemicals were obtained from either Sigma or Fisher. Radiolabeled chemicals were from American Radiolabeled Chemicals, and other isotopically labeled chemicals were from Cambridge Isotope Laboratories. Phospholipids were obtained from Avanti Polar Lipids. Cell culture media and supplies were from Invitrogen.

Chlamydia Strain and Propagation—C. trachomatis serovar L2 (strain 434/Bu) was propagated by standard infection through centrifugation protocol in HeLa cells grown in DMEM containing 10% fetal bovine serum (38) in 6-well tissue culture-treated plates (Corning). Mock infection controls for HeLa cells were performed by going through the same protocol but without C. trachomatis.

Phospholipid NMR—Eight plates of mock-infected or C. trachomatis-infected HeLa cells grown for 48 h after infection were collected for each NMR sample. The cells were scraped from the plate and washed twice with phosphate-buffered saline. The lipids were extracted using the Bligh and Dyer method (39). The NMR analysis was performed as described (40). Briefly, the lipid extracts were suspended into 500 μl of the cholate detergent system through vortexing and dispersion in a sonicating bath at 60 °C for 30 min. The cholate detergent system consisted of sodium cholate (10% w/v), EDTA (10% w/v), phosphonomethylglycine for internal standard (0.3 g/liter), and 20% D2O for deuterium field frequency locking. The pH was adjusted to 7.3 using sodium hydroxide. Continuous proton-decoupled 31P NMR spectra were recorded on a Bruker Ultrashield 400 Plus instrument at a probe temperature of 300 K, tuned to 161.98 MHz, using 5-mm high resolution NMR tubes. Spectra were obtained with a spectral width of 64102.56 Hz, 2.0 s relaxation delay, and 32,768 complex points in the time domain using simultaneous detection of real and imaginary components. Ten thousand scans were taken per sample. Chemical shifts are reported relative to the internal sample of [1,2,2,3,3,4,4,5,5-2H9]choline at 0 ppm. Phospholipids were identified by comparing the chemical shifts to phospholipid standards run under the same conditions. Experiments were performed in triplicate with average and standard deviations reported.

Chlamydia Labeling Experiments—Kinetic radioisotope labeling experiments were performed on C. trachomatis L2-infected HeLa cultures 18 h post infection. The cell cultures were washed with 1 ml of Hanks’ balanced salt solution (HBSS), and 1.5 ml of fresh DMEM with 10% FBS and 10 μM ethanamine supplement was added. The respective radiolabeled lipid precursors ([14C]choline, [14C]ethanolamine, and [14C]serine at 5 μCi per well and [14C]glucose at 25 μCi per well) were added to the culture and incubated for 8 h at 37 °C and 5% CO2. Cells were harvested and the lipids extracted (39), and the incorporation of radioactivity was measured by liquid scintillation counting (Tri-Carb 2910TR, PerkinElmer Life Sciences). Uninfected control HeLa cells were mock-infected but otherwise treated the same. Experiments were performed in triplicate with average and standard deviations reported. The distribution of [14C]glucose labeling into different lipid classes was determined by fractionating the radiolabeled lipid extracts on Silica Gel H thin layers (Analtech) developed with chloroform/methanol/acetic acid/water (50:25:8:4, v/v). The plate was dried and radioactivity quantified using a Bioscan AR-2000.

[14C]Choline incorporation into Renografin or Nonidet P-40 isolated C. trachomatis was determined by adding 5 μCi of [14C]choline per well at 18 h post-infection. [14C]Glucose incorporation was determined by resuspending the infected HeLa cells in DMEM with 10% FBS and 0.4 g/liter glucose plus 2.5 μCi of [14C]glucose per well at 18 h post-infection. The cultures were grown for an additional 30 h, and the cells were then processed via the Renografin or Nonidet P-40 protocol. The lipids were extracted from the harvested cells, and the amount of label incorporated was quantified by liquid scintillation counting. The distribution of [14C]glucose label in different lipid classes was analyzed via thin layer chromatography as described above. HeLa cells were plated at 12.5% confluence on 10-cm cell culture plates in DMEM with 10% FBS (10 ml) and 5 μCi of [3H]arachidonic acid per plate. The cells were allowed to grow to confluence over 3 days, and then cells from each 10-cm plate were split into 2 × 6-well plates. The cells in the 6-well plates were infected (or mock-infected) with C. trachomatis and grown for 48 wells. Then, the media were collected, and the cells were resuspended into HBSS and harvested. The radioactivity in the media was counted by liquid scintillation counting to determine the amount of [3H]arachidonic acid released into the media. The harvested cells were extracted, and the amount of radioactivity remaining in the cells was determined by liquid scintillation counting. The extracts were also chromatographed on Silica Gel H layers developed with 50:25:8:4 chloroform/methanol/acetic acid/water, and percentage of PE radioactivity in the phospholipid fraction was determined. The experiment was conducted in quadruplicate with each replicate consisting of three wells of material. For ethanol-1,1,2,2-d4-amine (D4-Etn) labeling experiments, C. trachomatis-infected HeLa cells were incubated in DMEM supplemented with 100 μCi D4-Etn at 18 h post-infection for 30 h. Cells were collected, and the lipids were extracted and analyzed by mass spectrometry.

Mass Spectrometry—Phospholipid molecular species fingerprints were determined using direct infusion electrospray ionization-mass spectrometry technology (41, 42). Mass spectrometry analysis was performed using a FinniganTM TSQ® Quantum (Thermo Electron, San Jose, CA) triple quadruple mass spectrometer. The lipid extracts were resuspended in 50:50 (v/v) chloroform/methanol with 1% formic acid. The instrument was operated in positive ion mode for PC and PE analysis and negative ion mode for PG and CL analysis. Ion source parameters were spray voltage 3500 V, capillary temperature 270 °C, and capillary offset 35 V, and the tube lens offset was set by infusion of the polyterosine tuning and calibration solution (Thermo Electron, San Jose, CA) in electrospray mode. Parameters for the analysis of PC are as follows: scan range, 600–900 m/z; scan time, 0.3 s; product mass, 184.1 m/z; collision energy, 40 V; peak width, Q1 and Q3 0.7 full width at half-maximum (FWHM); and Q2 CID gas, 0.5 mtorr. Parame-
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For PG and CL analysis, lipid classes were separated using a Discovery DSC-NH2 solid-phase extraction column (Supelco, Bellefonte, PA) (43). In brief, the column was conditioned with 8 ml of hexane, and lipid extract was added. Nonpolar lipids were eluted with 6 ml of 2:1 (v/v) chloroform/isopropanol alcohol; fatty acids were eluted with 6 ml of ether + 2% acetic acid; phosphatidylcholine and CL were eluted with 6 ml of methanol; and phosphatidylglycerol and cardiolipin were eluted with 6 ml of chloroform/methanol, 0.8 M sodium acetate 60:30:4.5 (v/v). Parameters for PG analysis are as follows: scan range, 600–900 m/z; scan time, 0.3 s; product mass, 153.0 m/z; collision energy, 45 V; peak width, Q1 and Q3 0.7 FWHM. For D4-PE analysis, lipids from L2-infected HeLa cells were separated on a Silica Gel G layers developed with chloroform/methanol/acetic acid/water (50:20:8.3, v/v). Parameters are as follows: scan range, 600–900 m/z; product mass, 141.0 m/z; scan time, 0.3 s; collision energy, 45 V; peak width, Q1 and Q3 0.7 FWHM; and Q2 CID gas, 0.5 mtorr.

For fatty acids scans, lipids from C. trachomatis-infected HeLa cells were separated using Silica Gel G layers developed with chloroform/methanol/acetic acid/water (50:20:8.3, v/v). The area of silica where PE migrated was scraped off the plate, and PE was extracted using the Bligh and Dyer method. The instrument was operated in negative ion mode. Parameters for PG analysis are as follows: scan range, 1200–1500 m/z; scan time, 0.5 s; peak width, Q1 0.7 FWHM. For D4-PE analysis, lipids from L2-infected HeLa cells were separated on a Silica Gel G layers developed with chloroform/methanol/acetic acid/water (50:20:8.3, v/v). Parameters are as follows: scan range, 1200–1500 m/z; scan time, 0.5 s; peak width, Q1 0.7 FWHM; and Q2 CID gas, 0.5 mtorr. Parameters for CL analysis are as follows: full scan, scan range, 1200–1500 m/z; scan time, 0.5 s; peak width, Q1 0.7 FWHM. For D4-PE analysis, lipids from L2-infected HeLa cells were separated on a Silica Gel G layers developed with chloroform/methanol/acetic acid/water (50:20:8.3, v/v). Parameters are as follows: scan range, 1200–1500 m/z; scan time, 0.5 s; peak width, Q1 0.7 FWHM.

For TD-MS analysis, lipids from L2-infected HeLa cells were collected by cell scraping, washed twice with HBSS, resuspended in 0.5% Nonidet P-40 (octyl phenoxypolyethoxylethanol, Sigma IGEPAL CA-630), and lysed with gentle sonication (three times for 20 s at 8 watts). The resulting solution was centrifuged at 500 × g for 15 min, and the supernatant was pelleted over a 40% sucrose cushion via centrifugation for 30 min at 20,000 × g. The pellet was resuspended in 0.1% Nonidet P-40 and centrifuged over 40% sucrose cushion two more times to collected the EB.

Molecular Biology—The plsE (CT807) and plsC (CT453) genes of C. trachomatis strain D/UW-3/Cx (NCBI Microbial Genomes Database) was optimized for expression in E. coli through GeneArt Gene Synthesis Technology (Life Technologies, Inc.). For the plsC gene, an NdeI restriction site was engineered at the 5′ of the gene with start codon in the NdeI site, and a His6 tag, stop codon, and an EcoRI restriction site were sequentially engineered at the 3′ of the gene. The plsC gene was cloned into the pPJ131 plasmid (a modified version of the pBlueScript plasmid with the multiple cloning site from pET28a) via the NdeI and EcoRI (New England Biolabs) restriction sites (5). A BamHI restriction site was engineered at the 5′ of the plsE gene, and a His6 tag, stop codon, and an EcoRI restriction site were sequentially engineered at the 3′ of the gene. The plsE gene was cloned into pFastBac1 plasmid (Life Technologies, Inc.) via the BamHI and EcoRI restriction sites.

PlsE Characterization and Assay—The pFastBac1-CtplsE vector was expressed in insect cells following protocols of the Invitrogen Bac-to-Bac Expression System and then purified via a standard nickel chelation chromatography. The fractions containing protein, as determined by the Bradford reagent (47), were collected and dialyzed against 20 mM Tris, pH 8.0, 10 mM EDTA, and 150 mM NaCl at 4 °C overnight. A pure protein (>95%) running at ~38 kDa (theoretical average mass of 38.7 kDa) with an N-terminal methionine was observed on a NuPAGE 10% bis-Tris gel (Life Technologies, Inc.). Approximately 5–10 mg of purified PIsE was purified per liter of culture. The oligomerization state of PlsE was determined by size exclusion chromatography on a calibrated Superdex 200 10/300 GL column (GE Healthcare) in 20 mM Tris, pH 8.0, and 200 mM NaCl compared with standards running on the same column. Enzymatic activity of PlsE was determined by incubating increasing concentrations of PlsE (0, 10, 20, 40, and 80 pmol) with [14C]Gro-3-P (50 μM), 16:0-ACP (50 μM), or 16:0-CoA (50 μM, Avanti Polar Lipids), 2 mM MgCl2, 0.1% Brij-58, and 100 mM Tris, pH 8.0, for 30 min at 37 °C. The reaction was extracted via the Bligh and Dyer method (39), and lysophosphatidic acid formation was quantified by liquid scintillation counting.

Synthesis of 16:0-ACP—E. coli ACP was used for the PlsE assay. Apo-ACP was purified as described previously (48) and converted into 16:0-ACP in a two-step reaction. Apo-ACP (100 μM), dithiothreitol (5 mM), MgCl2 (10 mM), coenzyme A (1 mM), and 10 μg per ml of Streptococcus pneumoniae ACP synthase was incubated in 100 mM Tris-HCl, pH 8.0, at 37 °C for 2 h. Then, a final concentration of ATP (10 mM), palmitic acid (160, 150 μM) in...
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| PL Class | Average | StDev |
|----------|---------|-------|
| PC       | 49.2%   | 1.5%  |
| PI       | 6.8%    | 1.3%  |
| PS       | 6.9%    | 0.4%  |
| LPC      | 3.3%    | 1.1%  |
| PE       | 19.3%   | 2.4%  |
| SM       | 9.9%    | 2.3%  |
| DHSM     | 1.9%    | 0.5%  |
| CL       | 2.8%    | 0.5%  |
| PG       | 0.0%    | 0.0%  |

| PL Class | Average | StDev |
|----------|---------|-------|
| PC       | 44.2%   | 1.2%  |
| PI       | 4.4%    | 0.4%  |
| PS       | 2.3%    | 0.4%  |
| LPC      | 2.5%    | 0.5%  |
| PE       | 31.3%   | 0.5%  |
| SM       | 8.7%    | 0.9%  |
| DHSM     | 1.9%    | 0.4%  |
| CL       | 3.3%    | 0.2%  |
| PG       | 1.5%    | 0.2%  |
Altered phospholipid content and metabolism in C. trachomatis-infected HeLa cells. The phospholipid composition of uninfected and C. trachomatis-infected HeLa cells was quantified by $^{31}$P NMR spectroscopy. A, representative $^{31}$P NMR spectrum of phospholipids extracted from uninfected HeLa cells 48 h after a mock infection. B, representative $^{31}$P NMR spectrum of phospholipids from HeLa cells 48 h after infection with C. trachomatis. The peaks were identified based on the $^{31}$P NMR spectra of phospholipid standards. The phospholipid composition was quantified by measuring the integral, and the results of triplicate biological experiments are shown in the table adjacent to the spectra. C, comparison of the incorporation of $[^{14}]$Etn into the lipid fraction of uninfected HeLa cell cultures (blue) to C. trachomatis-infected HeLa cultures (red). $[^{14}]$Etn-labeled extracts were fractionated by thin layer chromatography, and the proportion of label incorporated into the two major phospholipid classes (PC and PE) were determined. D, percentage of $[^{14}]$Etn incorporated into PC and PE in uninfected and C. trachomatis-infected cultures. E, comparison of the incorporation of $[^{14}]$C-choline into the lipid fraction of uninfected HeLa cells to C. trachomatis-infected HeLa cells. F, comparison of the incorporation of $[^{14}]$C-serine into the lipid fraction of uninfected HeLa cells and C. trachomatis-infected HeLa cells. Data were from triplicate biological experiments, and significance was determined using Student’s t test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. PL, phosphatidylcholine; DCHS, dihydrocholine; LPC, lysophosphatidylcholine.
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A. Uninfected - PC

B. Infected - PC

C. Uninfected - PE

D. Infected - PE

E. Uninfected - PG

F. Infected - PG

G. Uninfected - CL

H. Infected - CL
pared with uninfected cells (Fig. 3, A and B). In both cases, the most abundant PC species were 16:0/18:1, 16:0/16:1, 18:1/18:1, and 14:0/16:0. There was no evidence for branched-chain fatty acids in the PC molecular species, ruling out their incorporation into PC via a deacylation/reacylation pathway. In contrast, the PE molecular species profile was significantly different in the C. trachomatis-infected cultures (Fig. 3, C and D). Abundant PE species observed in uninfected HeLa cells contained polyunsaturated fatty acids (18:0/20:4 and 18:1/22:6). These major PE molecular species in uninfected cells became minor species in the C. trachomatis-infected HeLa cells. In the C. trachomatis infected HeLa cells, odd-number disaturated PE molecular species containing branched-chain fatty acids became predominant. The most abundant new PE species were 16:0/15:0 and 17:0/15:0. These data showed that bacterially derived branched-chain fatty acids were only detected in phospholipids predicted to be bacterially synthesized, but they were absent from host phospholipids. These data also showed that the contribution of C. trachomatis-derived PE to the total PE in the infected cultures was larger than the 31P NMR experiments implied (Fig. 2, A and B) because major HeLa cell PE molecular species containing polyunsaturated fatty acids were lost from the PE pool as a consequence of C. trachomatis infection.

PG was a phospholipid that was observed in infected HeLa cells that was not detected in uninfected HeLa cells (Fig. 2, A and B). The bioinformatic model posits that PG and PE would arise from a common PA precursor, and therefore these two phospholipids would be expected to have a similar molecular species profile. The major PG molecular species from uninfected HeLa cells contained unsaturated fatty acids (primarily 16:0/18:1 and 18:1/18:1) (Fig. 3E). The molecular species of PG isolated from C. trachomatis-infected HeLa cells (Fig. 3F) were dominated by the same saturated branched-chain fatty acid combinations that characterized the new PE molecular species appearing in C. trachomatis-infected cells (Fig. 3D). Host CL molecular species contained primarily unsaturated fatty acids (Fig. 3G). In contrast, the most abundant CL species in C. trachomatis-infected HeLa cells were saturated and contained between 60 and 65 total carbons (Fig. 3H). The most abundant CL species containing 62 carbons corresponded to the condensation of the most abundant PG molecular species (16:0/15:0, Fig. 3F). The data showed that the new PE, PG, and CL have molecular species profiles consistent with the bioinformatic model that predicts they arise from the same PA pool.

Host Phospholipids Were Not Remodeled during C. trachomatis Infection—C. trachomatis infection activates cPLA2, and the degraded phospholipids may be subsequently modified by putative C. trachomatis acyltransferases to create new molecular species (21, 53). The reduction in host PE molecular species containing polyunsaturated fatty acids in C. trachomatis-infected cultures was consistent with the degradation and/or remodeling of selected PE molecular species. Metabolic labeling experiments with D4-Etn were performed to determine whether the new PE molecular species in C. trachomatis-infected cultures were derived from remodeling of host PE. Host PE arises from either the CDP-Etn or PS decarboxylase pathways, whereas the bioinformatics model predicted that C. trachomatis only uses the PS decarboxylase route (Fig. 1). Thus, host PE was selectively labeled via the CDP-Etn pathway by growing C. trachomatis-infected HeLa cells in the presence of 100 μM D4-Etn. The PE molecular species containing normal and D4-Etn were identified by mass spectrometry. The molecular species profile of PE containing normal Etn (Fig. 4A) was the same as previously determined for C. trachomatis-infected cells (Fig. 3D). PE molecular species containing heavy D4-Etn consisted only of unsaturated host PE molecular species (Fig. 4B). There was no evidence for D4-Etn incorporation into the new saturated PE molecular species that arose in C. trachomatis-infected cell cultures (Fig. 4B). A minor 18:1/15:0 PE molecular species was consistently detected in C. trachomatis-infected cultures (Figs. 3D and 4A). The 18:1 fatty acid originated from the host and may arise from remodeling of the host 18:1/18:1 PE species. However, host 18:1/18:1 PE was the most abundant PE species labeled with D4-Etn, but isotopically labeled 18:1/15:0 PE was not detected (Fig. 4B) meaning that the PE backbone was of bacterial origin. Mass spectrometry was used to identify phospholipids containing a branched-chain fatty acid (15:0) produced by C. trachomatis FASII. The branched-chain fatty acid was only detected in the saturated PE molecular species arising in C. trachomatis-infected cells (Fig. 4C). We also detected a PG molecular species containing 15:0 (Fig. 4C). Significantly, PC did not contain 15:0. These data were consistent with the conclusion that the new PE molecular species found in C. trachomatis-infected cells arose from the de novo C. trachomatis biosynthetic pathway (Fig. 1) and not from the modification of pre-existing host PE.

The analysis of C. trachomatis-infected cells indicated that PE molecular species containing polyunsaturated fatty acids were selectively reduced during the infection. This point was investigated by pre-labeling HeLa cells with [3H]arachidonic acid and, after removing the label from the cells, comparing uninfected and C. trachomatis-infected HeLa cells to determine whether infection altered the metabolism of PE-containing [3H]arachidonic acid. There was a significant increase in the release of [3H]arachidonic acid into the medium and a concomitant decrease in [3H]arachidonic acid in the PE of C. trachomatis-infected cultures (Fig. 4D). These data indicated that the selective degradation of host polyunsaturated PE molecular species accounted for the alteration in the PE molecular species profile in C. trachomatis-infected cells (Fig. 3, C and D).

C. trachomatis Acyltransferases and PA Synthesis—Gro-3-P acyltransferase (PlsE) and 1-acyl-Gro-3-P acyltransferase
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C. trachomatis PlsC contained the HX4D motif found in other PlsC proteins and has homology to the S. aureus PlsC, which is also selective for anteiso-15:0 fatty acids (23% identity, 43% similarity, and e-value of 6e-13). The predicted function of C. trachomatis PlsC in PA synthesis was validated by complementation experiments using temperature-sensitive E. coli strain SM2-1 (plsC(Ts)) (54) to analyze PlsC function. The plasmids used were the empty vector and vectors expressing either C. trachomatis, E. coli, or S. aureus plsC. All three plsC genes restored the growth of strain SM2-1 at the nonpermissive temperature, but the empty plasmid did not (Fig. 5, D and E). While this work was underway, the CT807 gene was shown to complement strain SM2-1 (22). These results showed that the C. trachomatis PlsC was a 1-acyl-Gro-3-P acyltransferase that utilized acyl-ACP. PlsC substrate specificity can also be inferred from these experiments. E. coli PlsC selectively utilizes 16:1, an unsaturated fatty acid. Thus, the molecular species of PE produced in strain SM2-1 complemented with E. coli PlsC was 16:0/16:1 (Fig. 5F). In S. aureus, anteiso-15:0 is the primary fatty acid found in the 2-position (50), and complementation of strain SM2-1 with S. aureus PlsC

FIGURE 4. Origins of PE molecular species in C. trachomatis-infected cells. C. trachomatis-infected HeLa cells were grown in the presence of 100 µM D4-Etn, and the phospholipids were extracted 48 h after infection. The PE molecular species containing normal Etn or heavy D4-Etn were profiled by mass spectrometry. Spectra are representative examples from duplicate biological experiments. A, profile of PE molecular species that contained the normal isotope of Etn derived from either the CDP-Etn or PS decarboxylase pathways. B, profile of the PE molecular species that incorporated D4-Etn by the host CDP-Etn pathway. C, profile of the PE molecular species that contained a bacterially derived 15:0 fatty acid was determined by ESI-MS/MS. D, HeLa cells were prelabeled with [3H]arachidonic acid and washed to remove the extracellular label. The cells were then either mock-infected or infected with C. trachomatis and incubated for 48 h. The amounts of radioactivity released into the media and retained in cellular PE were determined by scintillation counting and thin layer chromatography. Data were from quadruplicate biological replicates, and the significance was determined using Student’s t test: ***, p < 0.001.
resulted in the formation of 16:0/14:0 PE molecular species (Fig. 5G). *S. aureus* PlsC incorporated a 14:0 fatty acid into the 2-position because it was the closest structural analogue to anteiso-15:0 (14-carbon chain with a methyl branch) synthesized by *E. coli* FASII (Fig. 5G). In strain SM2-1 complemented with the *C. trachomatis* PlsC, the molecular species
analysis showed the incorporation of 14:0 as did the strain complemented with \textit{S. aureus} PlsC (Fig. 5H), indicating that the substrate specificity of \textit{C. trachomatis} PlsC was similar to \textit{S. aureus} PlsC. These data were consistent with the predicted role for \textit{C. trachomatis} PlsC in placing anteiso-15:0 fatty acids into the 2-position during the synthesis of PA.

**Phospholipids Associated with Isolated \textit{C. trachomatis} EB—** We compared two established methods for \textit{C. trachomatis} isolation to address the host lipid contribution to \textit{C. trachomatis} cell membranes. The first method used a Renografin density gradient centrifugation to isolate \textit{C. trachomatis} EB (44), and the second employed a Nonidet P-40 detergent extraction step. The detergent extraction was developed to remove extraneous host cellular components, lyse immature RBs, and solubilize connected host membrane systems (46). Although the detergent step removed some \textit{C. trachomatis} outer membrane proteins, electron microscopy shows that detergent-extracted \textit{C. trachomatis} retained their intracellular contents and have intact inner and outer membrane systems (45, 55, 56). Uninfected and \textit{C. trachomatis}-infected HeLa cell cultures were labeled with \[^{14}C\]choline to quantify the proportion of host choline-containing phospholipids that were associated with Renografin- and Nonidet P-40-isolated \textit{C. trachomatis}. The amount of host PC recovered in the \textit{C. trachomatis} fraction was 1.33 ± 0.08% of the total \[^{14}C\]PC applied to the Renografin gradient (Fig. 6A). A similar experiment performed with lysates derived from \[^{14}C\]choline-labeled uninfected HeLa cells showed that 0.24 ± 0.01% of the \[^{14}C\]PC was located in the gradient fraction that would have contained \textit{C. trachomatis}. Contamination of the Renografin \textit{C. trachomatis} preparation with host PC was confirmed by these experiments. \textit{C. trachomatis} from \[^{14}C\]choline-labeled \textit{C. trachomatis}-infected HeLa cells were also isolated by the Nonidet P-40 method. In these experiments, 0.27% of the total \[^{14}C\]PC was found in the EB fraction, and a much smaller amount of label was recovered from the uninfected controls (Fig. 6A). These data indicated that Nonidet P-40 detergent extraction was more efficient at removing host lipids from \textit{C. trachomatis}.

The PC molecular species profile from Renografin-isolated \textit{C. trachomatis} (Fig. 6B) was similar to the profile of host PC (Fig. 3A). In contrast, the phosphocholine-containing molecular species detected in the Nonidet P-40-isolated \textit{C. trachomatis} were distinctly different. The SM in HeLa cells that was a minor species detected in the scan of total cellular PC (Fig. 3A) was highly enriched in the Nonidet P-40-isolated bacteria (Fig. 6C). The PE molecular species profiles were similar in both the Renografin and Nonidet P-40 isolation methods (Fig. 6, D and E). The amount of host PC/SM compared with bacterial PE/PG/CL was estimated by labeling infected HeLa cells with \[^{14}C\]glucose followed by the isolation of \textit{C. trachomatis} by the Nonidet P-40 method. A thin layer chromatography system was developed to clearly separate these two groups of phospholipids, and the distribution of radioactivity was determined (Fig. 6F). Approximately 15% of the labeled phospholipids associated with isolated \textit{C. trachomatis} were SM/PC, and 85% were of bacterial origin. One interpretation of these data could be that SM was an integral component of \textit{C. trachomatis} membranes. However, further corroborating evidence will be required to establish SM as an integral bacterial membrane component because it is possible we were detecting contaminating detergent-insoluble host SM in the detergent-washed \textit{C. trachomatis} preparation.

**Discussion**

Our results show \textit{C. trachomatis} produces the phospholipids required for the formation of its membrane systems using its own biosynthetic pathways described in Fig. 1. The membrane structures of \textit{C. trachomatis} are morphologically similar to free living Gram-negative bacteria with clearly defined inner and outer membrane systems (3, 36, 37), and \textit{C. trachomatis} cells possess the gene set found in free-living bacteria like \textit{E. coli} to generate the phospholipid components. Two differences are the absence of genes for unsaturated fatty acid synthesis and the use of an acyl-ACP-specific plant-like Gro-3-P acyltransferase. As in many bacteria, \textit{C. trachomatis} encodes a branched-chain ketoacid dehydrogenase and produces a mixture of saturated and branched-chain fatty acids (58). Thus, \textit{C. trachomatis} FASII is designed to supply the fatty acids detected in the new PE, PG, and CL molecular species produced in \textit{C. trachomatis}-infected cultures. The removal of host phospholipids may be difficult because \textit{C. trachomatis} cells are tightly associated with the host inclusion membrane by a structure termed the pathogen synapse (3, 36, 37). The existence of these intimate \textit{C. trachomatis}-inclusion membrane interactions may account for the higher level of host lipid associated with \textit{C. trachomatis} EB in the Renografin density gradient centrifugation isolation method than in the isolation method using a detergent extraction step. Regardless of whether there is a small contribution of host phospholipids to \textit{C. trachomatis} membranes, it is clear that \textit{C. trachomatis} uses its own biosynthetic apparatus to produce the bulk of the fatty acids and glycerophospholipids used to construct its membrane systems. Building blocks that are required to assemble these phospholipids (glucose, isoleucine and serine) are readily available from the medium via the host cell.

Our experiments provide analytical data that address the contributions of bacterial and host-derived phospholipids to \textit{C. trachomatis} membranes. Many experiments with fluorescent

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**FIGURE 5. \textit{C. trachomatis} acyltransferases catalyze phosphatidic acid synthesis.** A, His-tagged version of the putative \textit{C. trachomatis} PlsE was expressed in insect cells and purified to greater than 95% purity by affinity and size exclusion chromatography as judged by SDS-gel electrophoresis. B, elution position of PlsE on a calibrated Superdex 200 gel filtration column indicated a molecular mass of 37 kDa consistent with \textit{C. trachomatis} PlsE existing as a monomer in solution (calculated mass, 38.7 kDa). C, purified CTPsE catalyzed the conversion of \[^{14}C\]Gro-3-P into lysy-PA using 16:0-ACP as the acyl donor, but activity was not detected when 16:0-CoA was the acyl donor. D, growth of temperature-sensitive \textit{E. coli} strain SM2-1 (plcCTS)) transformed with the empty pJP131 plasmid (Control Plasmid) or its derivatives expressing either \textit{C. trachomatis} PlsC (CpTsE), \textit{E. coli} (EcpTsC), or \textit{S. aureus} (SaPlsC). Strains were grown at the permissive growth condition at 30 °C. E, complementation of growth on the nonpermissive temperature of 42 °C in the strain set described in D. F, PE molecular species profile of strain SM2-1 complemented with SaPlsC grown at 42 °C. H, PE molecular species profile of strain SM2-1 complemented with CTPsC grown at 42 °C. Spectra are representative examples from duplicate biological experiments, and the major molecular species are labeled on the panels.
probes summarized in recent reviews (13, 14) show how host membrane trafficking pathways are hijacked to assemble the inclusion membrane, and the association of these probes with C. trachomatis or the inclusion lumen led to the idea that host phospholipids are used to construct C. trachomatis membranes. These experiments did not quantify the contribution of host lipids to the total and rely on the probes reflecting the behavior of host phospholipid molecular species. SM is the pre-
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dominant host phospholipid detected in detergent-extracted C. trachomatis. The detection of SM in the detergent-extracted bacteria coupled with the association of fluorescent ceramide probes with individual C. trachomatis in vivo suggests that SM may be an integral component of C. trachomatis membranes. However, our analytical experiments are perhaps inconclusive because SM is enriched in detergent-extracted mammalian membranes (59–61), and the enrichment in SM detected in Nonidet P-40-isolated C. trachomatis may arise from contaminating host membranes containing SM that is refractory to detergent extraction. Alternatively, the analytical work may support the incorporation of host SM into C. trachomatis membranes, which may have an unappreciated role in its complex life cycle. Additional research will be needed to evaluate these alternatives.

The idea that C. trachomatis modifies host phospholipids with branched-chain fatty acids by a deacylation-reacylation pathway (17, 18, 21) is definitively ruled out by our experiments. Mass spectrometry of C. trachomatis-infected cultures shows that neither host PC nor PE is modified with bacterially derived branched-chain fatty acids. The recent report identifying a C. trachomatis gene as a lyso-PC acyltransferase that functions with a host acyl-CoA-binding protein to remodel PC following C. trachomatis infection (22) is also incompatible with the analytical data. The selective degradation of polyunsaturated PE molecular species and the release of arachidonic acid into the medium are consistent with the activation of cPLA$_2$ observed by others (21, 53, 62). However, cPLA$_2$ activation appears to be related to the cellular immunity response to chlamydial infection rather than participating in bacterial phospholipid synthesis. The consistent detection of a minor 18:1/15:0 PE molecular species in the C. trachomatis-infected cultures that does not arise from remodeling host PE indicates that host fatty acids are able to access the C. trachomatis acyltransferases. The activation and utilization of host fatty acids may occur by the acyl-ACP synthetase/2-acylglycerophosphoethanolamine acyltransferase system (CT775 + CT776 = aas). This enzyme system in E. coli activates fatty acids for their transfer to the 1-position of 2-acyl-lyso-PE via an ACP intermediate (9, 10), but it does not release acyl-ACP for use by other acyltransferases. Further work will be required to determine whether the C. trachomatis genes produce proteins with the same functions.

There is considerable re-organization of host intracellular membranes and lipid trafficking following C. trachomatis infection to construct the expanding inclusion membrane that grows as C. trachomatis numbers increase and almost reaches the size of the entire cell as the infectious cycle nears completion (63). PC, cholesterol, and SM are trafficked to the chlamydial inclusion to construct this specialized membrane (19, 23, 24, 26). The lower yields of infectious C. trachomatis EB observed when host lipid synthesis/trafficking are inhibited illustrate the importance of host lipid metabolism to maximizing the yield of C. trachomatis (17, 19, 23, 26). The effect of SM inhibitors shows that blocking SM synthesis does not stop C. trachomatis replication but leads to the premature rupture of the inclusion membrane releasing C. trachomatis into the host cytoplasm (23). These data argue that host lipid trafficking is required to maximize the number of infectious EB produced per cell, but it does not impact the fitness of individual C. trachomatis. In contrast, autonomous C. trachomatis phospholipid synthesis is vital to the differentiation, replication, and survival of individual C. trachomatis. The targeted inhibition of the FabI component of FASII arrests C. trachomatis in an RB-like state and prevents replication and the differentiation into infectious EB, illustrating the effectiveness of this approach (5). Thus, the essentiality of autonomous fatty acid and phospholipid synthesis opens the door to the development of new anti-FASII therapeutics to treat C. trachomatis.

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