Zinc Metalloproteinase ProA Directly Activates *Legionella pneumophila* PlaC Glycerophospholipid:cholesterol Acyltransferase*

Christina Lang[‡], Elena Rastew[‡], Björn Hermes[‡], Enrico Siegbrecht[‡], Robert Ahrends[§], Sangeeta Banerji[‡], and Antje Flieger[‡1]

From the [‡]Division of Bacterial Infections, Robert Koch-Institut, Burgstrasse 37, 38855 Wernigerode, Germany and [§]Institute of Analytical and Environmental Chemistry, Humboldt University Berlin, Brook-Taylor Strasse 2, 12489 Berlin Adlershof, Germany

Background: PlaC is a GDSL enzyme and the major GCAT secreted by *Legionella pneumophila*.
Results: The zinc metalloproteinase ProA processes and activates PlaC. Deletion of regions within a disulfide loop increased GCAT activity.
Conclusion: Inhibitory disulfide loop reduction/deletion by ProA activates PlaC GCAT.
Significance: Here we recognized the postexport GCAT activation mechanism essential for modification of typical eukaryotic sterols.

Enzymes secreted by *Legionella pneumophila*, such as phospholipases A (PLAs) and glycerophospholipid:cholesterol acyltransferases (GCATs), may target host cell lipids and therefore contribute to the establishment of Legionnaires disease. *L. pneumophila* possesses three proteins, PlaA, PlaC, and PlaD, belonging to the GDSL family of lipases/acyltransferases. We have shown previously that PlaC is the major GCAT secreted by *L. pneumophila* and that the zinc metalloproteinase ProA is essential for GCAT activity. Here we characterized the mode of PlaC GCAT activation and determined that ProA directly processes PlaC. We further found that not only cholesterol but also ergosterol present in protozoa was palmitoylated by PlaC. Such ester formations were not induced by either PlaA or PlaD. PlaD was shown here to possess lysophospholipase A activity, and interestingly, all three GDSL enzymes transferred short chain fatty acids to sterols. The three single putative catalytic amino acids (Ser-37, Asp-398, and His-401) proved essential for all PlaC-associated PLA, lysophospholipase A, and GCAT activities. A further four cysteine residues are important for the PLA/GCAT activities as well as their oxidized state, and we therefore conclude that PlaC likely forms at least one disulfide loop. Analysis of cleavage site and loop deletion mutants suggested that for GCAT activation deletion of several amino acids within the loop is necessary rather than cleavage at a single site. Our data therefore suggest a novel enzyme inhibition/activation mechanism where a disulfide loop inhibits PlaC GCAT activity until the protein is exported to the external space where it is ProA-activated.

Legionella pneumophila resides in aquatic habitats and replicates in diverse species of protozoa. When inhaled by suscep-

tible humans, the pathogen causes a severe form of pneumonia, termed Legionnaires disease (1–3). Enzymes secreted by *Legionella*, such as phospholipases A (PLAs),[2] may contribute to the establishment of the disease and subsequent lung tissue destruction. So far, three different PLA families, namely the patatin-like, PlaB-like, and GDSL enzymes together with at least 15 distinct proteins, have been identified in *L. pneumophila* (4, 5).

Patatins were originally identified as a family of plant proteins with acyl hydrolase activity fulfilling important functions in signal transduction processes (6). Bacteria also possess patatin-like proteins. Ten to 11 patatin-like protein genes are typically found in the genome of *L. pneumophila* (*patA* to *patK*). Both bacterial patatin-like proteins and eukaryotic patatins are characterized by a catalytic dyad consisting of a nucleophile serine embedded into the typical lipase consensus motif G*X*S*X*G and an aspartate that is often found in a DGG motif (5, 7).

PlaB shows no significant protein homology to any established lipase/phospholipase family and is the most prominent cell-associated PLA/lysophospholipase A (LPLA) of *L. pneumophila*. PlaB homologues are yet uncharacterized proteins especially found in water bacteria. Instead of the characteristic G*X*S*X*G motif, PlaB constitutes the designated THSTG motif surrounding the catalytic serine. Aspartate and histidine, representing the other two members of the catalytic triad, are embedded into the uncommon motifs GSDGVV and SHS, respectively (8).

Three *L. pneumophila* proteins homologous to the "prototype" of GDSL enzymes, *Aeromonas salmonicida*/*Aeromonas hydrophila* SatA, are coded in *L. pneumophila* genomes and were designated PlaA, PlaC, and PlaD (4, 5, 9–15). GDSL

---

[2] The abbreviations used are: PLA, phospholipase A; LPLA, lysophospholipase A; GCAT, glycerophospholipid:cholesterol acyltransferase; DPPG, 1,2-dipalmitoylphosphatidylglycerol; DPPC, 1,2-dipalmitoylphosphatidylcholine; AEC, anion exchange chromatography; ESI, electrospray ionization; CP, cholesterol palmitate; CPP, cholesterol propionate.

TABLE 1

Overview of strains used in this study

L.pn., *L. pneumophila*; term., terminal.

Organism	Mutation(s)/plasmid	Tags	Selection marker(s)	Ref.
L.pn. Corby	Wild type			27
L.pn. Corby	*plaA* knock-out		Km^R	This study
L.pn. Corby	*plaC* knock-out		Km^R	This study
L.pn. Corby	*plaD* knock-out		Km^R	This study
L.pn. Corby	*proA* knock-out		Km^R	This study
L.pn. 130b	Wild type			Strain ATCC BAA-74
L.pn. 130b	*plaC* knock-out		Km^R	25
L.pn. 130b	*proA* knock-out		Km^R	Strain AA200 (30)
E. coli BL21	pet28a (+)	C-term. His$_6$ tag	Km^R	Novagen
E. coli BL21	pMMB2002		Cm^R	23
E. coli BL21	pER13 = pBADTOPO *plaA*$_{Corby}$	C-term. His$_6$ tag	Amp^R	This study
E. coli BL21	pMY6 = pMMB2002 *plaC*$_{130b}$		Cm^R	This study
E. coli BL21	pCL14, pER14 = pet28a (+) *plaC*$_{Corby}$	C-term. His$_6$ tag	Km^R	This study
E. coli BL21	pER31 = pGEX-6-P1 *plaC*$_{Corby}$(−SP^a)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL83 = pGEX-6-P1 *plaC*$_{Corby}$(−SP)-Strep	N-term. GST tag/C-term. Strep tag	Amp^R	This study
E. coli BL21	pCL70 = pGEX-6-P1 *plaC*$_{Corby}$S37N(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL71 = pGEX-6-P1 *plaC*$_{Corby}$ D398N (−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL72 = pGEX-6-P1 *plaC*$_{Corby}$ H401N(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL73 = pGEX-6-P1 *plaC*$_{Corby}$ C343N(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL74 = pGEX-6-P1 *plaC*$_{Corby}$ C388N(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL75 = pGEX-6-P1 *plaC*$_{Corby}$ C415N(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL76 = pGEX-6-P1 *plaC*$_{Corby}$ C427N(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL77 = pGEX-6-P1 *plaC*$_{Corby}$ Δ364–75(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL78 = pGEX-6-P1 *plaC*$_{Corby}$ Δ361–79(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL79 = pGEX-6-P1 *plaC*$_{Corby}$ Δ358–79(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL80 = pGEX-6-P1 *plaC*$_{Corby}$ Δ343–88(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pCL85 = pGEX-6-P1 *plaC*$_{Corby}$ Δ347–84(−SP)	N-term. GST tag	Amp^R	This study
E. coli BL21	pER7 = pET160 *plaD*$_{Corby}$	N-term. His$_6$ tag	Amp^R	This study
E. coli BL21	pCL15 = pet28a (+) *proA*$_{Corby}$	C-term. His$_6$ tag	Km^R	This study

^{a} (−SP), constructs cloned without sequence coding for the putative signal peptide (amino acids 1–24).

enzymes possess a conserved GDSL signature motif harboring the nucleophile Ser, which together with an Asp and a His (D*XX*H motif) form the catalytic triad (16, 17). The GDSL proteins share five conserved blocks of amino acid homology, belong to the SGNH hydrolases, and differ in several properties (folding structure, proximity of Ser nucleophile to the N terminus, and close localization of catalytic Asp and His) from the well characterized α/β-hydrolases (17, 18). GDSL family members are found in many bacteria as well as in higher plants and show among others lipase, LPLA, PLA, and/or glycerophospholipid:cholesterol acyltransferase (GCAT) activities (16, 17, 19).

PlaA is the major secreted LPLA of *L. pneumophila* and contains a predicted signal peptide (20, 21). Because of the absence of PlaA in the culture supernatant of a *L. pneumophila* type II secretion mutant, it is most likely exported via the Lsp type II pathway (20, 22, 23). During *L. pneumophila* host cell infection, PlaA promotes membrane destabilization of the *Legionella*-containing vacuole and activation of the host cell death pathway when the type IVB (Dot/Icm)-secreted effector SdhA is lacking (24).

Lipolytic activity of the PlaD enzyme has not yet been conclusively shown (4, 5). It contains a C-terminal appendage of about 170 amino acids that is missing in PlaA and PlaC. A signal peptide is not predicted for PlaD, implying either a cell-associated localization or its export by a pathway different from the Sec system (4, 5).

PlaC contains a predicted signal peptide and shows 27% sequence identity to PlaA, 24% to PlaD, and 22% to *A. salmonicida* SatA. A *L. pneumophila plaC* knock-out mutant exhibits an approximately 20% reduction in secreted PLA and a 10% reduction in secreted LPLA activities (25). Furthermore, *L.*

pneumophila possesses secreted GCAT activity (21). *plaC* but not *plaA* knock-out mutants completely lost their ability to transfer long chain fatty acids from dipalmitoylphospholipids to cholesterol. Therefore, PlaC is the major secreted GCAT of *L. pneumophila* with PLA/LPLA activities (25). Furthermore, the presence of GCAT activity within *L. pneumophila* culture supernatant depends on the intact Lsp type II secretion system and the type II-secreted zinc metalloproteinase ProA (22, 23, 26). This suggested proteolytic activation of PlaC GCAT activity.

Here we aimed to clarify whether ProA directly (and by which mechanism) activates PlaC GCAT activity. Our data suggest a novel enzyme inhibition/activation mechanism where a disulfide loop inhibits PlaC GCAT and full PLA activity until the protein is exported to the external space and ProA-activated.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*L. pneumophila* sg1 strain 130b (ATCC BAA-74) was used to purify the PlaC GCAT-activating factor via anion exchange (AEC) and gel filtration chromatography as described previously (25). *L. pneumophila* sg1 strain Corby (27) was used for all other experiments, including knock-out mutant construction as well as for recombinant gene expression in *Escherichia coli* strains DH5α and BL21. The strains used are listed in Table 1 and were grown on buffered charcoal yeast extract agar and in buffered yeast extract broth (both for *Legionella*) or on Luria-Bertani (LB) agar and in LB broth (both for *E. coli*) as described previously (25, 28, 29).

DNA Techniques and Sequence Analysis—*E. coli* DH5α or BL21 was used for the propagation of recombinant plasmid

TABLE 2
Overview of primers used in this study
term., terminal.

Plasmid	Gene	Tag	Primer name	Primer sequence
pER13	*plaA*(−SP[a])	C-term. His tag	BADPlaAf	5′-GGTTTCATGAAACTATTAGCAT-3′
			BADPlaAr	5′-AAACTCTTCATTCTCGGCGAA-3′
pER31	*plaC*(−SP)	N-term. GST tag	PlaC_ohneSP_Eco	5′-TGAATTCATGACGTCTATTCCAGTGAAA-3′
			PlaC_XhoI_rev	5′-AGCTCGAGGTATTAGGCGGACTCTGGCAT-3′
pCL83	*plaC*(−SP)-Strep	N-term. GST tag	pGEX_Strep_fw	5′-CCGCAGTTCGAAAAATAATACCTCGAGCGGCCG-3′
			PlaC_Strep_rv	5′-GTGGCTCCAGCTAGCGGCGGACTCTGGCAT3–3′
pCL14, pER14	*plaC*	C-term. His tag	PlaC_EcoRI	5′-TGAATTCTTATGATCCAAAACAACAGGG-3′
			PlaC_XhoI	5′-AGCTCGAGGTATTGGGCGGACTCTGGCAT-3′
pCL70	*plaC*$_{S37N}$(−SP)	N-term. GST tag	PlaC_Serin_f	5′-AGTGGTGTTTGGAGACAACTTGTCTGATAACGGTAATA-3′
			PlaC_Serin_rev	5′-TATTACCGTTATCAGACAAGTTGTCTCCAAACACCACT-3′
pCL71	*plaC*$_{D398N}$(−SP)	N-term. GST tag	PlaC_D398N_fw	5′-TATTTATTCTGGAATGAAATTCATCCA-3′
			PlaC_D398N_rv	5′-TGGATGAATTTCATTCCAGAATAAATA-3′
pCL72	*plaC*$_{H401N}$(−SP)	N-term. GST tag	PlaC_H401N_fw	5′-TGGGATGAAATTAATCCAACCACGAGA-3′
			PlaC_H401N_rv	5′-TCTCGTGGTTGGATTAATTTCATCCCA-3′
pCL73	*plaC*$_{C343N}$(−SP)	N-term. GST tag	PlaC_C343N_fw	5′-GAAGGATGCCAATATCGATGTGA-3′
			PlaC_C343N_rv	5′-TCACATCGATATTGGCATCCTTC-3′
pCL74	*plaC*$_{C388N}$(−SP)	N-term. GST tag	PlaC_C388N_fw	5′-CTATCACATGAATAGTGAACCAG-3′
			PlaC_C388N_rv	5′-CTGGTTCACTATTCATGTGATAG-3′
pCL75	*plaC*$_{C415N}$(−SP)	N-term. GST tag	PlaC_C415N_fw	5′-TTATGAGGTTAATTTGGCGTTGG-3′
			PlaC_C415N_rv	5′-CCAACGCCAAATTAACCTCATAA-3′
pCL76	*plaC*$_{C427N}$(−SP)	N-term. GST tag	PlaC_C427N_fw	5′-CGAGGTAACTAACAAAATGCCAG-3′
			PlaC_C427N_rv	5′-CTGGCATTTTGTTAGTTACCTCG-3′
pCL77	*plaC*$_{ΔA364–75}$(−SP)	N-term. GST tag	PlaC_del375fw	5′-TTGAGCTCGTAGTTACGAGCAAATGGAG-3′
			PlaC_del364rv	5′-AAGAGCTCAAGCAATTTGCTCCAGGT-3′
pCL78	*plaC*$_{Δ361–79}$(−SP)	N-term. GST tag	PlaC_del379fw	5′-CCAGGTGAAAATAACTATCACATG-3′
			PlaC_del361rv	5′-ACGAGCAAATGGAGAGTGAAT-3′
pCL79	*plaC*$_{Δ358–79}$(−SP)	N-term. GST tag	PlaC_del379fw	5′-CCAGGTGAAAATAACTATCACATG-3′
			PlaC_del358rv	5′-AGAGTGAATGAATGCATTGAAC-3′
pCL80	*plaC*$_{Δ343–88}$(−SP)	N-term. GST tag	PlaC_del388fw	5′-AGTGAACCAGAAGACTATTTATTC-3′
			PlaC_del343rv	5′-GGCATCCTTCACATTATAAAAAC-3′
pCL85	*plaC*$_{Δ347–84}$(−SP)	N-term. GST tag	PlaC_del384fw	5′-TATCACATGTGTAGTGAACCAGAA-3′
			PlaC_del347rv	5′-CACATCGATACAGGCATCCTT-3′
pMY4	P$_{plaC}$ *plaC*		gdsl2_a1_f	5′-TGCTTAAAAACCGCTCTGGA-3′
			gdsl2_b1_r	5′-TTAACGGCATATTGGGTGAA-3′
pER7	*plaD*	N-term. His tag	PlaD_fTopo	5′-CACCGCAAATATCATGGCCCAAAAA-3′
			PlaD_d1_r	5′-AGGGACGCATTAAAACTTACCAG-3′
pSB2	*plaD*		gdsl3_a1_f	5′-TTTTGCAAGTGGATTAGGTGA-3′
			gdsl3_b1_r	5′-ACGTCCGATTTGTATGAGTG-3′
pCL15	*proA*	C-term. His tag	ProA_BamHI_fw	5′-ATGGATCCATGCACCCAAATTATTAT-3′
			ProA_XhoI_rv	5′-ATCTCGAGATCGACATAACAAGATTG-3′
pAF14	*proA*:Kn^R		ProA3a_fw	5′-ATAACAAACCTGGGCACTCC-3′
			ProA4a_rv	5′-GACACGATCGCAGGCTATTA-3′

[a] (−SP), without signal peptide.

DNA with backbones of the following vectors: pMMB2002 (23), pet28a(+) (Novagen), pBADTOPO (Invitrogen), pBCKS (Stratagene), pGEX-6P-1 (GE Healthcare), pGEM-T Easy (Promega GmbH), and pET160 (Invitrogen). Genomic and plasmid DNA were prepared, amplified, and sequenced according to standard protocols. Primers were obtained either from Tib Molbiol or from Eurofins MWG Operon. Restriction enzymes were purchased from New England Biolabs. Foreign DNA was introduced into *E. coli* or *L. pneumophila* strains by electroporation with an Invitrogen cell porator according to the manufacturer's specifications as described earlier (25). Nucleotide and translated protein sequences were analyzed using the DNASTAR package, the pedant website, the NCBI website, ExPASy, multiple sequence alignment by Florence Corpet, ClustalW multiple alignment, and BPROM.

Cloning of L. pneumophila plaA, plaC, plaD, and proA into Vectors for Recombinant Expression in E. coli—*L. pneumophila* Corby *plaA* (*lpc1811*) was cloned into the pBADTOPO vector resulting in pER13 (C-terminal 6×His$_6$ tag). *L. pneumophila* Corby *plaC* (*lpc3121*) was cloned into the pet28a(+) and pGEX-6-P1 vectors, resulting in plasmids pCL14 (C-terminal His$_6$ tag) and pER31 (N-terminal GST tag), respectively. To introduce mutations into the *plaC* gene, the pER31 vector was

mutated by means of the QuikChange site-directed mutagenesis kit (Stratagene) with the primers listed in Table 2, resulting in pCL70–pCL76. To create PlaC disulfide loop deletion mutants or a GST-PlaC construct, pER31 was amplified with primers listed in Table 2 and ligated, resulting in pCL77–pCL80 and pCL85 or pCL83, respectively. *L. pneumophila* 130b *plaC* was cloned into the pGEM-T Easy vector, yielding pMY4. After digestion of pMY4 by SpeI and SacII, the insert was cloned into pBCKS, yielding in pMY5. This vector was restricted by SacI and KpnI, and the insert was ligated into pMMB2002, yielding pMY6. *L. pneumophila* Corby *plaD* (*lpc0558*) or *proA* (*lpc2877*) was cloned into pET160, yielding pER7 (N-terminal His$_6$ tag), or pet28a(+), resulting in pCL15 (C-terminal His$_6$ tag), respectively (for primers, see Table 2).

L. pneumophila GDSL Enzyme Knock-out Mutant Construction—Plasmids pAF3 (21), pBH5 (25), pBH4 (this study), and pAF14 (this study) containing the *plaA*, *plaC*, *plaD*, and *proA* genes, respectively, disrupted or partially replaced by a Km^R cassette were used to introduce a Km^R gene insertion mutation into the chromosome of strain Corby via allelic exchange. To assemble pBH4, *L. pneumophila* 130b *plaD* was cloned into the pGEM-T Easy vector, yielding pSB2 (for primers, see Table 2). After pSB2 restriction by means of HindIII and

NdeI, the insert was cloned into the pBCKS vector, yielding pBH3. pBH3 was digested with AfeI, which cleaves *plaD* at position 806 bp, to insert the Kn^R gene cassette, yielding pBH4. To generate pAF14, *proA*:Kn^R was cloned from an *L. pneumophila* 130b *proA* knock-out mutant (AA200; kindly provided by N. P. Cianciotto) into pGEM-T Easy (for primers, see Table 2) (30). Knock-out mutants were generated by natural transformation and homologous recombination (21, 31). PCR and/or Southern blot analysis was used to examine Km^R *Legionella* for the respective mutation.

Preparations of Culture Supernatants and Cell Lysates—For assessment of hydrolytic activities, bacteria were harvested at the end of exponential growth (if not stated otherwise at an OD_{660} of 2.0–2.1). Culture supernatants were obtained by centrifugation for 5 min at $5000 \times g$ and in some instances were subsequently concentrated by ultrafiltration with an exclusion size of 10 kDa. Cell lysates were produced as described previously (21, 32). Culture supernatants and cell lysates were either tested immediately for enzymatic activities or were stored overnight at 4 °C. To prove recombinant overexpression of proteins in *E. coli*, 5 μl of the cell lysate was used for SDS-PAGE with Coomassie Blue staining. For Western blot analysis, *Legionella* supernatants were complemented with 10 mM 1,10-phenanthroline (metalloproteinase inhibitor) before concentration.

Culture Supernatant Fractionation for Partial Purification of the PlaC-activating Factor—Five liters of culture supernatant of *L. pneumophila* 130b *plaC* mutant was 15-fold-concentrated by ultrafiltration with an exclusion size of 30 kDa, and the resulting retentate was directly applied to a 6-ml Resource-Q column (Amersham Biosciences) pre-equilibrated in buffer containing 20 mM Tris-HCl (pH 7.5) for AEC. AEC was carried out at 25 °C, and a gradient of 0–1 M sodium chloride in equilibration buffer was used for elution of proteins followed by gel filtration using a prepacked HiLoad 26/60 Superdex 200 column (Amersham Biosciences). Finally, 10-ml fractions were collected and used for SDS-PAGE with silver staining as well as for evaluation of protease activity by means of paracaseinate precipitation on casein-buffered starch yeast extract agar plates or azocasein hydrolysis and were stored at 4 °C (20, 33–36).

Purification of Recombinant ProA—Recombinant periplasmic ProA was isolated by osmotic shock (37) from *E. coli* BL21(pCL15). The presence of protease activity was determined as described above (34, 36). To obtain purified ProA, the periplasmic fraction was diluted 1:10 in 20 mM Tris-HCl (pH 9) equilibration buffer and applied to a 1-ml Resource-Q column (Amersham Biosciences) for AEC. AEC was carried out at 25 °C, and a gradient of 0–1 M sodium chloride in equilibration buffer was used. Fractions of interest were combined and concentrated, and buffer exchange with 20 mM Tris-HCl (pH 8) was performed by means of Amicon-15 filter devices (Millipore) and centrifugation. The presence of a single ProA-His protein band of 38 kDa was verified with SDS-PAGE and Coomassie staining.

Overexpression of Recombinant PlaA-His, GST-PlaC Variants, PlaC-His, and His-PlaD and Purification of GST-PlaC-Strep and PlaC-His—For expression of recombinant PlaA-His (from pER13), GST-PlaC variants (from pER31, pCL70 – 80/83/85), PlaC-His (from pCL14, pER14), and His-PlaD (from pER7),

E. coli BL21 cultures were grown at 37 °C to an A_{600} of 0.8. The GST-PlaC variant-expressing cultures were induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside and transferred to 18 °C for 16–17 h. PlaA-His, PlaC-His, and His-PlaD-expressing cultures were induced with 0.2% arabinose for PlaA-His or 1 mM isopropyl 1-thio-β-D-galactopyranoside for all others and collected after 3–4 h of incubation at 37 °C. Cell lysates for lipolytic activities were produced as described previously (21, 32). For the purification of GST-PlaC-Strep (from pCL83), the cell pellet was collected and resuspended in 100 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 1 mM EDTA. Homogenization was performed using an Emulsi Flex C3 (Avestin) applying 25,000–30,000 p.s.i. The soluble fraction was isolated by centrifugation at $13,000 \times g$ at 4 °C for 60 min. GST-PlaC-Strep was purified using Strep-Tactin® Superflow® high capacity resin (IBA) according to the manufacturer's instructions. To obtain PlaC-Strep, GST-PlaC-Strep bound to the resin was cleaved by PreScission protease (GE Healthcare) according to the manufacturer's instructions. Cells for the purification of PlaC-His (pER14) were resuspended in 20 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole (pH 7.4) and incubated with lysozyme (10 mg/ml) for 30 min on ice followed by ultrasonication and DNase treatment steps. After centrifugation at $13,000 \times g$ at 4 °C for 30 min, PlaC-His was purified using a HisTrap HP column (Amersham Biosciences) according to the manufacturer's instructions and concentrated by Amicon-15 filter devices (Millipore).

Assays for Lipolytic Activities—Lipolytic (PLA, LPLA, lipase, and GCAT) activities were detected as described previously (21, 25, 38) by using the lipid substrates 1-monopalmitoyllysophosphatidylcholine, 1-monopalmitoyllysophosphatidylglycerol, 1-monopalmitoylglycerol, monopalmitoyllysophosphatidylethanolamine, dipalmitoylphosphatidylserine, dipalmitoylphosphatidylethanolamine, 1,2-dipalmitoylphosphatidylglycerol (DPPG; used in most cases as an acyl donor for GCAT detection), 1,2-dipalmitoylphosphatidylcholine (DPPC), cholesterol, and ergosterol (used in amounts comparable with cholesterol). To activate GCAT in cell lysates with expressed recombinant PlaC, 100-μl reaction mixtures (lipid suspension and bacterial product) were incubated with 2–6 milliunits of different ProA preparations, such as culture supernatant from a *L. pneumophila plaC* mutant or ProA from the periplasmic fraction of BL21(pCL15). All lipids, including standards for thin layer chromatography (TLC), were obtained from Sigma or Avanti Polar Lipids. Detection of reaction products, such as free fatty acids and sterol esters, was performed by lipid extraction and subsequent TLC or by quantitative detection of free fatty acids as described earlier (21, 25).

Western Blot Analysis—Samples were separated on a 12.5% SDS-polyacrylamide gel (39). Proteins were transferred to a nitrocellulose membrane. Primary polyclonal rabbit antibody against PlaC (generated by BioGenes against recombinantly expressed and purified PlaC-Strep) was added at a dilution of 1:1000. α-Rabbit linked to G-horseradish peroxidase (Sigma) was used as a secondary antibody at a dilution of 1:10,000. Proteins were detected by chemiluminescence (ECL kit, Amersham Biosciences).

Proteolysis of PlaC and Peptide Analysis by Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry (Nano-LC/ESI-MS/MS)—Recombinant purified PlaC-His derived from BL21(pER14) was applied to SDS-PAGE, and the ~51-kDa protein band was cut out, destained (10% acetic acid, 30% ethanol, 60% water (v/v/v)), rinsed with 100 μl of 50 mM ammonium hydrogen carbonate buffer, and subsequently dehydrated with 100 μl of 100% acetonitrile until the fragment became colorless. Next, the fragment was quickly rinsed with ultrapure water, dried in a SpeedVac, and incubated overnight with 0.6 μg of ProA (purified from *L. pneumophila* supernatant via AEC) in 40 mM Tris-HCl (pH 7.5), 1 μM ZnCl$_2$. Trypsin proteolysis of ProA-digested PlaC-His and determination of generated PlaC peptides via nano-LC/ESI-MS were carried out according to a published protocol (40). The ProA-specific cleavage sites in PlaC were determined by exact peptide mass analysis and fragmentation of the ionized peptides applying collision-induced fragmentation. Peptide identifications were performed using the Mascot search engine (reference genome sequences, *L. pneumophila* Corby and 130b). The precursor and fragment mass tolerances were set to 0.002 and 0.5 Da, respectively.

Protease-profiling Microarray—For identification of ProA cleavage sites, a peptide microarray (JPT Peptide Technologies) was used. For that purpose, phosphotyrosine-labeled peptide octamers with five overlapping amino acids from the C-terminal part of *L. pneumophila* 130b PlaC (amino acids 300–433) were synthesized. In short, the peptide microarray (containing three identical subarrays) was incubated with 350 μl of purified *L. pneumophila* 130b ProA (1 unit/ml) in 1× TBS with 10 μM ZnCl$_2$ for 15 h at 37 °C. As a negative control, 10 mg/ml BSA was applied to an identical microarray. Next, the microarray slides were washed five times with double distilled water followed by treatment with fluorescence-labeled anti-phosphotyrosine antibody. Finally, fluorescence signal intensities of each spot were determined, and the mean value for signal intensities of the spots with identical peptides for the image of control experiment (A) and from images subsequent to ProA incubation (B) was calculated. The calculated ratio (A/B) displayed cleavage efficiency. The highest values (>1) indicated that the spots displaying the peptides were cleaved most effectively by ProA. The assay procedure was carried out according to the manufacturer's instruction.

RESULTS

Chromatographic Separation of the GCAT-activating Factor—Because *L. pneumophila proA* knock-out mutant does not transfer palmitic acid to cholesterol and therefore does not exhibit GCAT activity in culture supernatants, it is likely that zinc metalloproteinase ProA directly or indirectly promotes GCAT activation (25). To identify the protein responsible for PlaC GCAT activation, we performed AEC fractionation of *L. pneumophila plaC* mutant culture supernatant, which contains the activating factor but not PlaC. We subsequently incubated the fractions with cell lysates of an *E. coli* clone expressing PlaC but not showing GCAT activity without respective activation (25). Fractions 7–14/15 and especially fractions 8 and 9 indeed induced GCAT activity as shown by the formation of choles-
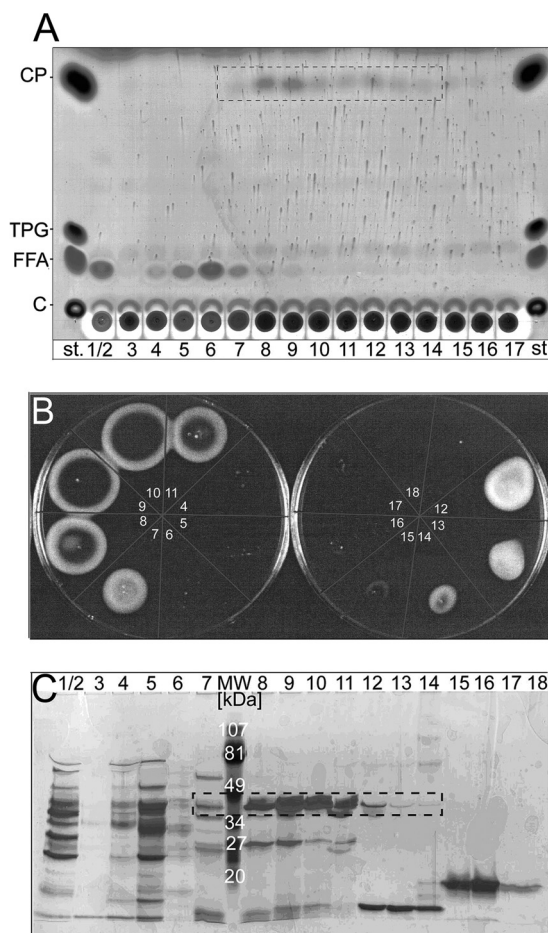

FIGURE 1. **Chromatographic separation of an *L. pneumophila* ~38-kDa protease as the GCAT-activating factor of PlaC.** *A*, AEC fractions (1–17) from culture supernatant of *L. pneumophila* 130b *plaC* mutant were assayed for GCAT activation of *plaC*-expressing DH5α (pMMB2002 *plaC* = pMY6). DH5α(pMY6) cell lysates were incubated with the respective AEC fraction, DPPG/cholesterol, and 2 mM ZnCl$_2$ for 20 h at 37 °C. Subsequently, lipids were extracted and subjected to TLC. Accumulation of cholesterol palmitate indicates GCAT activity in fractions 7–14/15 (*dashed frame*). Results are representative for at least two additional experiments. *st.*, standard; *TPG*, tripalmitoylglycerol; *FFA*, free fatty acids; *C*, cholesterol. *B*, AEC fractions 4–18 were applied to an agar plate containing casein and incubated for 8–18 h at 37 °C. Precipitation of paracaseinate indicates the presence of proteinase activity in the fractions showing GCAT activation. *C*, silver-stained reducing SDS-PAGE of AEC fractions showed enrichment of an ~38-kDa protein in the GCAT-activating fractions. Results are representative of at least one additional experiment.

terol palmitate (CP) when the mixtures were incubated with DPPG and cholesterol, thereby highlighting the successful fractionation of the GCAT-activating factor (Fig. 1*A*). Fractions 1–6 and 16–17 did not show CP generation, but instead especially fractions 1/2 and 4–7 showed a marked release of fatty acids, indicating the presence of secreted *L. pneumophila* PLAs (Fig. 1*A*) (4, 5, 25). Next, the fractions were tested for protease activity on casein agar, and protease-positive fractions 7–15 were exactly overlapping the fractions inducing cholesterol acylation (Fig. 1*B*). Reducing SDS-PAGE showed the consistent presence of a prominent ~38-kDa protein in the fractions of interest especially in fractions 8–11 (Fig. 1*C*). The 38-kDa protein was N-terminally sequenced, and the sequence "EKVQA" was determined that corresponds to mature ProA starting from amino acid 208 after cleavage of the
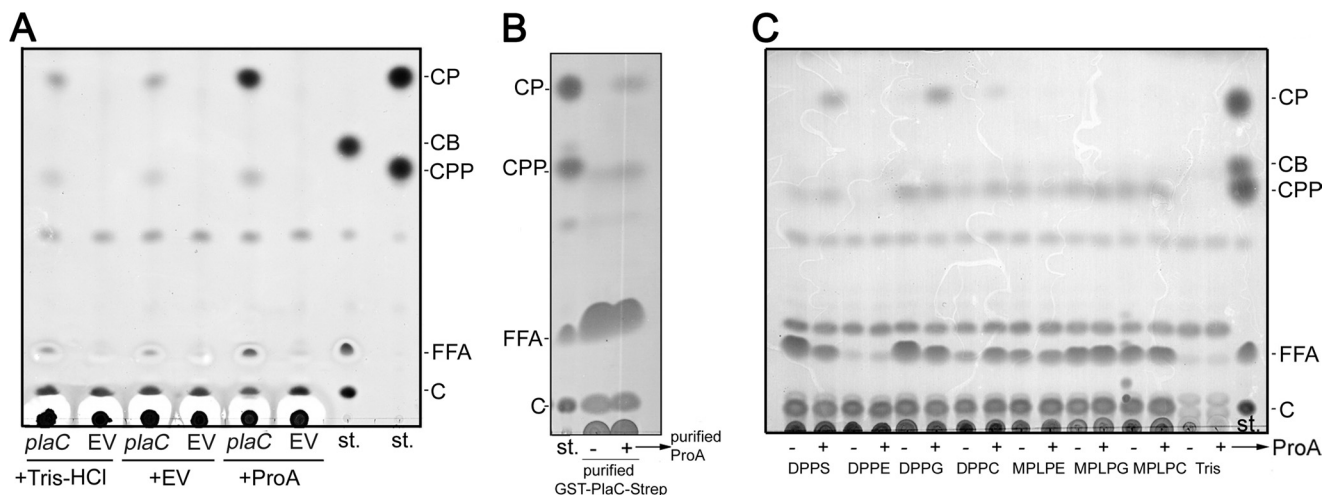
FIGURE 2. *L. pneumophila* **zinc metalloproteinase ProA directly activates PlaC GCAT activity transferring both palmitic and propionic acid to cholesterol and analysis of acyl donor substrate specificities.** *A, E. coli* BL21 cell lysates from clones expressing *plaC* (BL21(pet28a *plaC* = pCL14)) or harboring the empty vector (*EV*) (BL21(pet28a)) were incubated with DPPG/cholesterol and supplemented with Tris-HCl as the buffer control, the periplasmic fraction of BL21(pet28a) as the empty vector control, or the protease-active periplasmic fraction of *E. coli* expressing *proA* (BL21(pet28a *proA* = pCL15)). *B*, recombinantly expressed and purified GST-PlaC-Strep was incubated with DPPG/cholesterol and supplemented with Tris-HCl or recombinantly expressed and purified ProA-His. *C*, for determination of donor substrate specificities of GCAT activity, *L. pneumophila* Corby *proA* mutant 10-fold-concentrated late logarithmic phase culture supernatant was incubated with different diacyl- and lysophospholipids and cholesterol and supplemented either with Tris-HCl or protease-active periplasmic fractions of BL21(pet28a *proA* = pCL15). After 3 (*B*) or 16 h (*A* and *C*) of incubation, lipids were extracted and analyzed for the formation of cholesterol esters by means of TLC. All results shown are representative of at least two additional experiments. *st.*, standard; *CB*, cholesterol butyrate; *C*, cholesterol; *MPLPC*, 1-monopalmitoyllysophosphatidylcholine; *MPLPG*, 1-monopalmitoyllysophosphatidylglycerol; *MPLPE*, monopalmitoyllysophosphatidylethanolamine; *DPPS*, dipalmitoylphosphatidylserine; *DPPE*, dipalmitoylphosphatidylethanolamine.

signal peptide (amino acids 1–24) and the N-terminal propeptide (amino acids 25–207) (41).

ProA Directly Activates PlaC GCAT Activity—As judged from our biochemical analysis, it was likely that ProA directly activated PlaC GCAT activity. To confirm that observation, *L. pneumophila proA* and *plaC* were cloned into vector pet28a(+), resulting in C-terminal His$_6$-tagged gene fusions. Subsequently, the periplasmic fraction of *E. coli* BL21 expressing *proA* was incubated together with cell lysates of the *plaC*-expressing *E. coli* strain. The *plaC*-expressing *E. coli* clones or the empty vector controls alone showed only very minor or no GCAT activity. Only when *plaC*- and *proA*-expressing bacterial products were combined was a marked increase in CP generation observed (Fig. 2*A*). Also, recombinant purified GST-PlaC-Strep led to cholesterol acylation when incubated with purified ProA-His (Fig. 2*B*). In addition to the CP spot, another compound was formed due to PlaC activity (Fig. 2, *A* and *B*). This spot, running to a lower height than CP during TLC analysis, was also mentioned in earlier reports describing *L. pneumophila* GCAT activity expressed both naturally in *L. pneumophila* and recombinantly in *E. coli*. There it was designated "unknown substance" whose development depends on the presence both of cholesterol and PlaC; therefore, it might represent another cholesterol ester (21, 25, 33). We tested several defined cholesterol esters with different acyl chain lengths, such as cholesterol propionate (CPP) and butyrate, for their running behavior and found that the additional compound represents CPP (Fig. 2, *A* and *B*). To summarize, we found that PlaC GCAT is directly activated by ProA and that it also transfers fatty acids with a shorter chain length.

Acyl Donor Substrate Specificities of L. pneumophila GCAT—To assess the nature of the phospholipid acyl donor for *L. pneumophila* GCAT activity and its dependence upon activation by

ProA, we monitored CP generation after incubation of *L. pneumophila* Corby *proA* mutant culture supernatant (containing secreted not GCAT-activated PlaC) with cholesterol and different diacyl- and lysophospholipids, such as dipalmitoylphosphatidylserine, dipalmitoylphosphatidylethanolamine, DPPG, DPPC, monopalmitoyllysophosphatidylethanolamine, 1-monopalmitoyllysophosphatidylglycerol, and 1-monopalmitoyllysophosphatidylcholine. Here we show that only incubations with diacylphospholipids dipalmitoylphosphatidylserine and DPPG as well as to a lesser extent with DPPC and concomitant addition of recombinant ProA developed CP (Fig. 2*C*). This means that *L. pneumophila* GCAT activity preferentially transfers palmitic acid from dipalmitoylphosphatidylserine, DPPG, and DPPC to cholesterol, and it depends on enzyme activation by ProA. In contrast, development of CPP was observed for a variety of diacyl- and lysophospholipids and did not seem to be strictly dependent on ProA. This therefore suggests that enzymes in addition to PlaC may be responsible for transferring fatty acids with shorter chain lengths (Fig. 2*C*). We also performed experiments with 16:0 18:1 diacylphosphatidylglycerol and 16:0 18:1 diacylphosphatidylcholine as well as *Acanthamoeba* and U937 cell lysates (the latter two without external sterol addition) and observed that ProA-activated PlaC uses a variety of acyl lipid donors for sterol acylation but not unesterified fatty acids, such as propionic and palmitic acids (data not shown).

PlaC Is the Major L. pneumophila GCAT Transferring Palmitic Acid to Both Cholesterol and Ergosterol, and Together with PlaA and PlaD It Transfers Short Chain Fatty Acid to Sterols— It has been shown earlier that secreted GCAT activity of a *L. pneumophila plaC* knock-out mutant was negligible with respect to CP generation and that *plaA* does not contribute to that activity (25). Here we examined the three *L. pneumophila* single GDSL enzyme knock-out mutants for their GCAT activ-
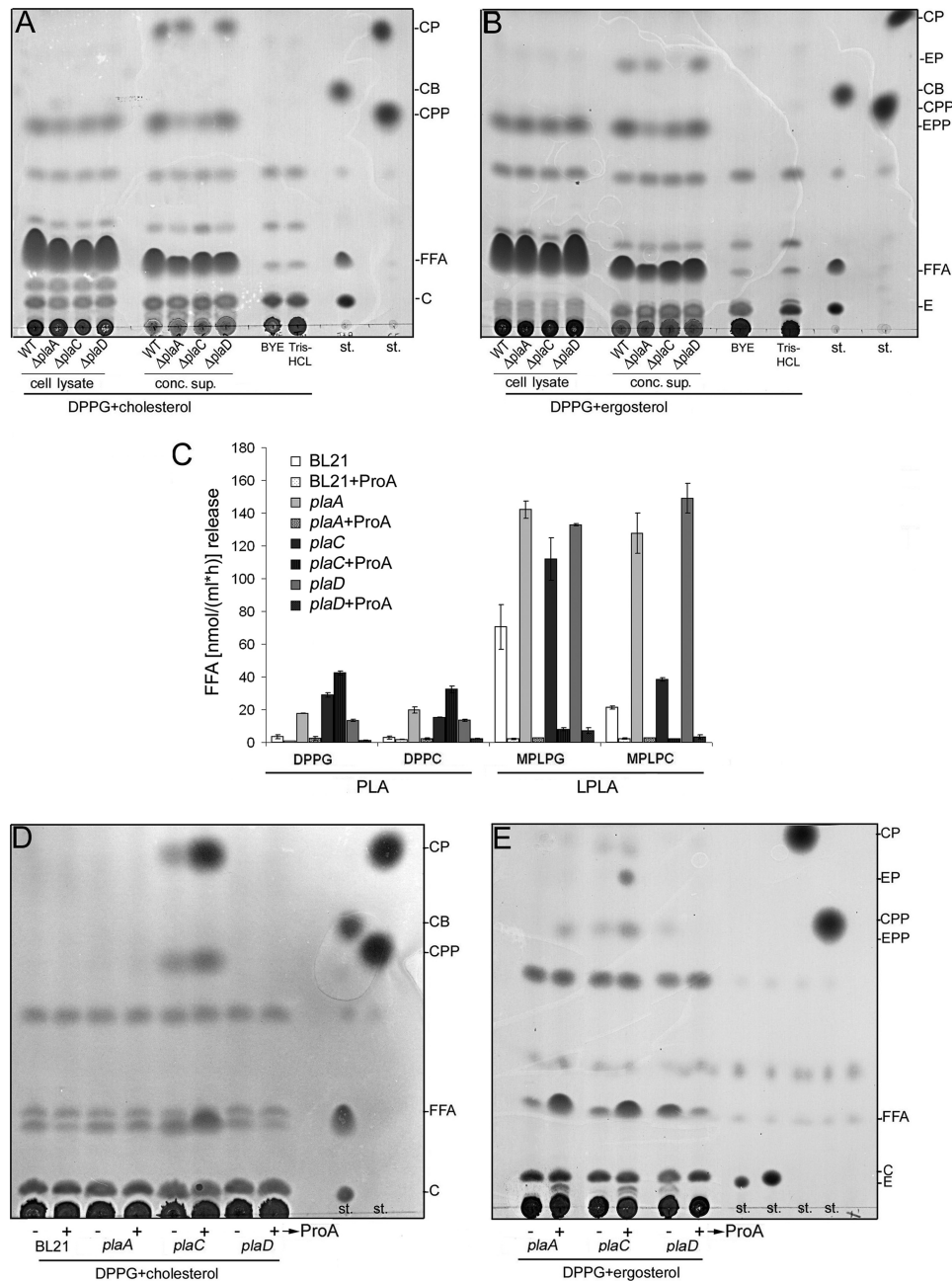
FIGURE 3. **PlaC is the major *L. pneumophila* GCAT transferring palmitic acid to both cholesterol and ergosterol, and together with PlaA and PlaD it transfers propionic acid to sterols.** Formation of different cholesterol (*A*) or ergosterol esters (*B*) by late logarithmic phase cell lysates and 8-fold-concentrated culture supernatants from *L. pneumophila* Corby wild type *versus* single GDSL protein knock-out mutants was studied after 16-h incubation at 37 °C with DPPG/sterol, lipid extraction, and TLC. Cell lysates of *E. coli* clones expressing *plaA*, *plaC*, and *plaD* (BL21(pBADTOPO *plaA* = pER13), BL21(pet28a *plaC* = pCL14), and BL21(pET160 *plaD* = pER27)) were incubated with DPPG, DPPC, 1-monopalmitoyllysophosphatidylglycerol (*MPLPG*), and 1-monopalmitoyllysophosphatidylcholine (*MPLPC*) with Tris-HCl or the periplasmic fraction of BL21(pet28a *proA* = pCL15) to determine PLA/LPLA activities via quantification of FFA (*C*). The results represent the means and standard deviations of duplicate cultures and three reactions each and are representative of three independent experiments. The *E. coli* preparations were further incubated with DPPG/cholesterol (*D*) or DPPG/ergosterol (*E*) with Tris-HCl or the periplasmic fraction of BL21(pet28a *proA* = pCL15) for 16 h, and then lipids were extracted and analyzed for sterol ester formation by means of TLC. All results shown are representative of at least one additional experiment. *st.*, standard; *CB*, cholesterol butyrate; *C*, cholesterol; *E*, ergosterol; *BYE*, buffered yeast extract; *EP*, ergosterol palmitate; *EPP*, ergosterol propionate.

ity with respect to CP and CPP development. To that end, we incubated cell lysates and concentrated culture supernatants of wild type *L. pneumophila* and single GDSL enzyme knock-out mutants with DPPG and cholesterol. Fig. 3*A* confirms that PlaC is the major secreted GCAT inducing CP production, whereas PlaA and PlaD do not contribute to this. However, because both *plaA* and *plaC* mutant culture supernatants showed reduced generation of CPP, we conclude that PlaA and PlaC transfer

short fatty acids (such as propionic acid) to cholesterol (Fig. 3*A*). Interestingly, generation of CPP was also induced by cell lysates of the wild type and all mutants. Therefore, *L. pneumophila* may contain another cell-associated enzyme, different from the three GDSL enzymes introduced here, that promotes cholesterol acylation.

L. pneumophila is predominantly an environmental bacterium that successfully replicates in protozoa, such as amoebae.

Because amoebae do not contain cholesterol but instead a variety of sterol species, including ergosterol (4, 25, 42–45), we were interested to know whether the *Legionella* GDSL enzymes acylate this potential acceptor lipid. We therefore incubated cell lysates and concentrated culture supernatants of *L. pneumophila* wild type and single GDSL enzyme knock-out mutants with DPPG and ergosterol. A result very similar to the above described cholesterol acylation was observed here; *i.e.* for the wild type supernatants, a compound spot that likely represents ergosterol palmitate and a spot likely representing ergosterol propionate were detected. Only the *plaC* mutant culture supernatant exhibited severely reduced production of ergosterol palmitate, and the *plaA* and *plaC* mutants exhibited reduced development of ergosterol propionate (Fig. 3*B*). The cell lysates of all tested strains showed the presence of a so far unknown cell-associated activity responsible for esterification of sterols (both ergosterol and cholesterol) (Fig. 3*B*).

To confirm the mutant studies and to get further insight into the *L. pneumophila* GDSL enzyme activities, *plaA*, *plaC*, and *plaD* were expressed in *E. coli*. All three genes were found to be expressed as shown by the presence of PLA/LPLA activities with and without addition of recombinant ProA (Fig. 3*C*). Recombinantly expressed PlaA and PlaD showed some PLA activity but major LPLA activity as already known for PlaA (21). For both enzymes, PLA and LPLA activities were reduced by ProA addition. Mainly, LPLA activity and some PLA activity were also found to be associated with the clone expressing *plaC*. As shown before, PlaC-dependent PLA activity but not LPLA activity increased by concomitant incubation with ProA (Fig. 3*C*) (25). Next, clones expressing the three genes were tested for GCAT activity. PlaC possessed activity for transfer of palmitic acid and propionic acid to both cholesterol and ergosterol that was activated by ProA (Fig. 3, *D* and *E*). The generation of sterol palmitate was not observed for *plaA*- and *plaD*-expressing clones; however, both induced generation of ergosterol propionate. Interestingly, in the case of PlaA, this ester was only formed with ProA, and in the case of PlaD, a weak spot of ergosterol propionate was observed only without ProA incubation (Fig. 3*E*). From those experiments, we conclude that not only PlaC but also PlaA and PlaD may transfer fatty acids to host cell sterols, and therefore all three enzymes possess acyltransferase activity. In this context, it seems that PlaA and PlaD may have a preference for the transfer of short chain fatty acids, such as propionic acid, whereas PlaC in addition transfers long chain fatty acids, such as palmitic acid.

PlaC Processing Depends on ProA—We have shown that ProA directly activates PlaC GCAT activity. Now it was of interest to determine whether PlaC is indeed processed by ProA. Therefore we analyzed the protein size of PlaC present in the culture supernatant of *L. pneumophila* wild type *versus* a *proA* mutant. Whereas in the wild type a protein of about 36 kDa was detected in the culture supernatant after reducing SDS-PAGE and Western blotting, in a *proA* mutant, an approximately 40–45-kDa protein was found (Fig. 4*A*). The unprocessed protein present in the *proA* mutant likely represents PlaC without a signal peptide (calculated mass, 47 kDa). To further evaluate the nature of processing, the PlaC protein sizes were compared under non-reducing SDS-PAGE. Here no con-
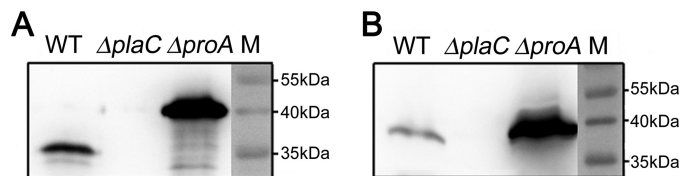

FIGURE 4. **Secreted PlaC is processed by ProA in *L. pneumophila*.** 10-fold-concentrated culture supernatants of *L. pneumophila* Corby wild type, *plaC* mutant, and *proA* mutant were applied to reducing (360 mM β-mercaptoethanol) (*A*) and non-reducing (*B*) SDS-PAGE, Western blot analysis, and detection with an α-PlaC antibody. Results are representative of at least one additional experiment. *WT*, wild type; *M*, molecular weight marker.

siderable difference in the protein size was observed (Fig. 4*B*). It was further found that after overexpression of PlaC-Strep in *L. pneumophila* wild type and affinity extraction of PlaC-Strep from the culture supernatant protein bands of ~38–39 and ~8–9 kDa, corresponding to processing at about amino acids 360–370, were detected after reducing SDS-PAGE, whereas only the full-length 47-kDa protein was found in a *proA* mutant (data not shown). This suggests processing within a disulfide loop where a disulfide bond still keeps the separated protein chains connected. PlaC contains four cysteine residues in its C-terminal half, and those might form disulfide loops involved in GCAT activation. But before clarifying the importance of cysteines, we aimed to characterize essential catalytic amino acids.

The Individual Members of the Putative Catalytic Triad Ser-37/Asp-398/His-401 Are Essential for the PlaC-associated GCAT and PLA Activities—Amino acid homology to *A. hydrophila* SatA and the presence of defined protein regions characteristic of GDSL enzymes and SGNH hydrolases within PlaC suggested the importance of Ser-37, Asp-398, and His-401 for enzymatic activity (Figs. 5 and 6*A*) (15, 19, 46, 47). To determine the significance of these three amino acids for PlaC PLA and GCAT activities, point mutations (S37N, D398N, and H401N) were introduced in GST-PlaC (Table 1). The constructs were expressed in *E. coli* and tested for the above mentioned activities. Fig. 6*B* shows that only the wild type but not the mutated construct possesses GCAT activity. PLA activity of the mutants was severely reduced compared with the wild type using DPPG as substrate (Fig. 6*C*), although the proteins were expressed at similar levels (Fig. 6*D*). Our data verify that Ser-37, Asp-398, and His-401 likely constitute the catalytic triad and that PlaC possesses identical catalytic residues for both GCAT and PLA activities.

Cysteines Cys-343, Cys-388, Cys-415, and Cys-427 Are Essential for PlaC-associated PLA and GCAT Activities—It has been shown that the GCAT activity of *A. salmonicida* SatA requires activation by a bacterial serine proteinase (48). Additionally, it was published that trypsin is able to activate *A. hydrophila* SatA GCAT expressed in *A. salmonicida* by means of processing the protein between two cysteines (Cys-225 and Cys-281), forming a disulfide bridge (49). *L. pneumophila* PlaC contains four cysteines in the C-terminal region that may form one or two disulfide bridges and thereby may assure integrity of the catalytic triad after possible proteolytic cleavage (Fig. 6*A*). Indeed, the program DiANNA predicted disulfide bridge formation (*a*) between the PlaC residues Cys-343 and Cys-388 and (*b*) the residues Cys-415 and Cys-427 (50). Insulin, chymotrypsinogen, prothrombin, and diverse bacterial toxins like the diphtheria

organism	name	block I		block II		block III		block IV		block V	
Aeromonas salmonicida	SatA	27	IVMF**GDSL**SDTG	37	LTIANEAEGGAT	37	VILWVGANDYL	26	NGAKQILLFNLPD	128	FW**D**QV**H**PT 24
Legionella pneumophila	PlaA	23	IVVF**GDSL**SDNG	41	SHLLDYAFGGAG	34	FVIWIGANNYL	26	KGAKHILVLNLPD	104	FF**D**LV**H**PT 25
Legionella pneumophila	PlaC	30	IVVF**GDSL**SDNG	108	EVYLNKAFGGSW	53	YFIYSGSNDYI	34	AGARRFVIMGIPH	123	FW**D**EI**H**PT 30
Legionella pneumophila	PlaD	10	LVVL**GDSL**SDRG	98	RFARFYCEGGLT	49	VVEWSGANDLI	78	KYKELGIPVNLSV	53	FW**D**DV**H**PT 170

FIGURE 5. **Conserved domains and catalytic triad (*bold*) of *L. pneumophila* Corby GDSL enzymes PlaA, PlaC, and PlaD compared with SatA of *A. salmonicida*.** The *digits* highlight the number of amino acids before and after the conserved regions.

toxin are good examples of enzymes that undergo processing and concomitant activation in between their disulfide bridge-forming residues (51–55) Therefore, to get insight into the importance of the four cysteine residues (Cys,343, Cys,388, Cys,415, and Cys,427), we incubated the wild type *plaC*-expressing clone with increasing amounts of the reducing agent dithiothreitol (DTT) and observed that GCAT activity for transfer of both palmitic and propionic acid was inhibited by 50 mM DTT (Fig. 6*E*). Additionally, the four cysteines were individually mutated in GST-PlaC and expressed in *E. coli*. Fig. 6, *F, G,* and *H,* illustrate that after mutation of Cys-343, Cys-388, Cys-415, and Cys-427, although expressed at a similar level, both GCAT and PLA activities were severely reduced or even completely lost as compared with that of the wild type construct. Collectively, these data imply that disulfide bridge formation between Cys-343 and Cys-388 as well as Cys-415 and Cys-427 may be essential for GCAT activity.

Determination of ProA-specific Cleavage Sites in PlaC: Disulfide Loop Reduction/Deletion Independently of ProA Activates PlaC-dependent PLA and GCAT Activities—Three different experimental strategies were followed to identify the potential ProA cleavage site within PlaC. Strategy 1 used an array of phosphotyrosine-labeled overlapping peptides of 8 amino acids, representing the last 134 amino acids (amino acids 300–433) of PlaC. Cleavage of a specific peptide was detected by comparison of spot intensities from identical peptides on chips incubated with ProA or BSA, respectively. One unambiguous cleavage site was found between Gln-366 and Tyr-367 located within the potential loop region between Cys-343 and Cys-388 (Fig. 7*A*). An additional, not clearly defined, potential cleavage site was found between the amino acids Phe-315 and Asn-319. Processing at this site, *i.e.* in front of the potentially disulfide bridge-forming cysteines, however, would separate the three members of the catalytic triad. Because we have shown that the lack of even one of the triad renders the enzyme inactive, we attribute the cleavage rather to the nature of peptides used in the assay and therefore did not include it for further analysis. No cleavage was observed in the region between Cys-415 and Cys-427. Strategy 2 purified denatured PlaC, which was digested with ProA and trypsin, and the resulting peptides were mapped by means of LC/ESI-MS. Here three major cleavage sites within the potential Cys-343 and Cys-388 loop, namely between Tyr-363 and Val-364, Gln-366 and Tyr-367, and Gln-372 and Tyr-373, were detected (Fig. 7*A*). The second proposed cleavage site (Gln-366 and Tyr-367) was also found by strategy 1. Additional cleavage was observed between Asp-314 and Phe-315, Phe-315 and Leu-316, Leu-316 and Phe-317, and Phe-317 and Val-318. This cleavage region matches a region also identified in strategy 1, but the site was not further analyzed due to the expected inactivation of the enzyme. Because several potential cleavage sites were observed for the region between Cys-343

and Cys-388 but no potential cleavage sites were observed between Cys-415 and Cys-427, we focused on a more detailed analysis of the first region. In strategy 3, five single (V364G, Q366A, Y367F, Y367W, and Y367A), five triple (P358A/F359A, R361A/N362A/Y363A, V364A/L365A/Q366A, Y367A/T368A/Q369A, and Y373A/R374A/D375A), and five double triple (P358A/F359A + Y367A/T368A/Q369A, P358A/F359A + V364A/L365A/Q366A, R361A/N362A/Y363A + Y367A/T368A/Q369A, V364A/L365A/Q366A + Y373A/R374A/D375A, Y367A/T368A/Q369A + Y373A/R374A/D375A) amino acid changes especially in the potential cleavage sites were applied to the region between Cys-343 and Cys-388; however, none of the PlaC mutants showed defective GCAT activation (data not shown). We then introduced deletions of different lengths within the loop structure, *i.e.* from amino acids 364 to 375, 361 to 379, 358 to 379, 347 to 384, and 343 to 388 (Fig. 7*A*). Except the latter deletion, all others showed a very interesting enzymatic phenotype: instead of the expected loss of ProA-induced activation of GCAT, these constructs possessed extraordinarily high GCAT and PLA activities even without activation by ProA (Fig. 7, *B* and *C*). GCAT activation of those deletion mutants was completely independent of ProA. This was observed despite the detection of comparable or even lower amounts of the respective proteins (Fig. 7*D*). This implies that reduction/deletion of the potential loop region, for example via proteolytic cleavage at several sites, rather than cleavage at a single specific site induces GCAT activity.

When an alignment of the *L. pneumophila* PlaC Cys-343/Cys-388 loop region with homologous proteins was performed, *Legionella* ssp. PlaC-like GDSL proteins were found to contain similar regions, whereas several cyanobacterial GDSL proteins and *Arabidopsis thaliana* extracellular lipase EXL4 lack the domain (Fig. 7*E*). This suggests that *Legionella* bacteria might possess specific means for postexport activation of GCAT and PLA activities by the described ProA-dependent mechanism (Fig. 8).

DISCUSSION

We postulate a novel enzyme activation mechanism by which cells may control a *per se* toxic enzymatic activity until it is released from the cell (Fig. 8). Such a mechanism might be of extraordinary importance to prevent damage of *Legionella* via its PlaC-dependent PLA activity that targets phospholipids typically contained in prokaryotes, such as phosphatidylglycerol or as shown for some intracellular bacteria phosphatidylcholine (56–58). Although the here described ProA-dependent increase in PlaC-derived PLA after expression in *E. coli* was not as prominent as seen for GCAT, we showed earlier that secreted PLA is reduced by two-thirds in a *L. pneumophila proA* knock-out mutant compared with the wild type (20).
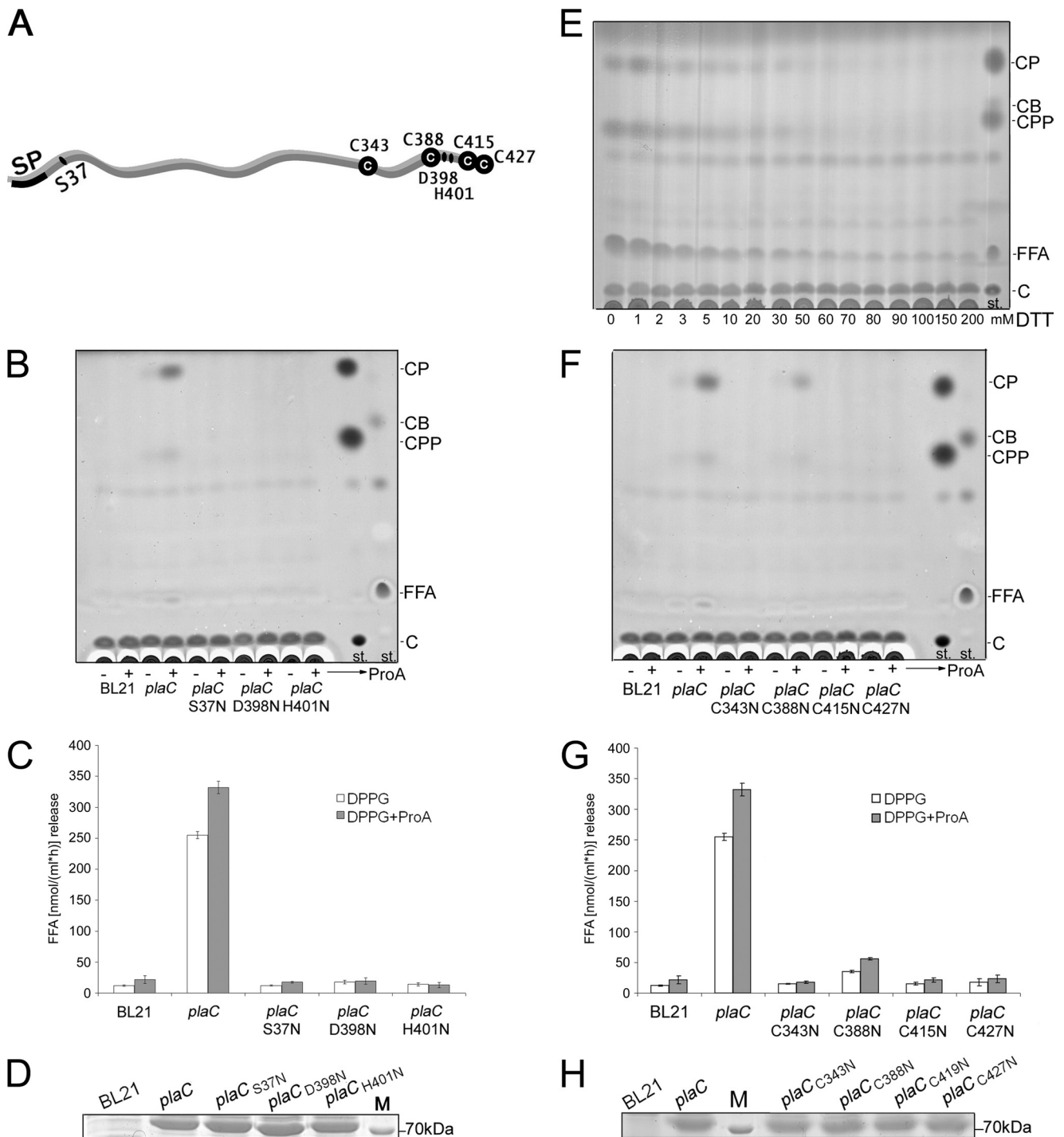
FIGURE 6. **The individual members of the putative catalytic triad Ser-37/Asp-398/His-401 and cysteines Cys-343, Cys-388, Cys-415, and Cys-427 are essential for the PlaC-associated GCAT and PLA activities.** The localization of the three catalytic amino acids, the four cysteine residues, and the predicted signal peptide (*SP*) of *L. pneumophila* PlaC is depicted in *A*. Cell lysates of *E. coli* clones expressing *plaC* (BL21(pGEX *plaC* = pER32)); the catalytic triad mutants (BL21(pGEX *plaC*_S37N = pCL70), BL21(pGEX *plaC*_D398N = pCL71), and BL21(pGEX *plaC*_H401N = pCL72)); or C343N, C388N, C415N, and C427N mutants (BL21(pGEX *plaC*_C343N = pCL73), BL21(pGEX *plaC*_C388N = pCL74), BL21(pGEX *plaC*_C415N = pCL75), and BL21(pGEX *plaC*_C427N = pCL76)) were incubated with DPPG/cholesterol with Tris-HCl or the periplasmic fraction of BL21(pet28a *proA* = pCL15) for 3 h, and then lipids were extracted and analyzed for sterol ester formation by means of TLC (*B* and *F*). The *E. coli* preparations were further incubated solely with DPPG supplemented with Tris-HCl or the periplasmic fraction of BL21(pet28a *proA* = pCL15) to determine PLA/LPLA activities via quantification of FFA (*C* and *G*). The results represent the means and standard deviations of duplicate cultures and three reactions each and are representative of three independent experiments. Comparable expression of the different *plaC* constructs was shown by detection of the overexpressed 74-kDa GST-PlaC protein in reducing SDS-PAGE and Coomassie Blue staining (*D* and *H*). Cell lysates of BL21(pGEX *plaC* = pER32) were incubated with DPPG/cholesterol supplemented with the periplasmic fraction of BL21(pet28a *proA* = pCL15) and with different concentrations of the reducing agent DTT for 3 h. Subsequently, the lipids were extracted and analyzed for sterol ester formation by means of TLC (*E*). All results shown are representative of at least one additional experiment. *st.,* standard; *CB*, cholesterol butyrate; *C*, cholesterol, *M*, molecular weight marker.
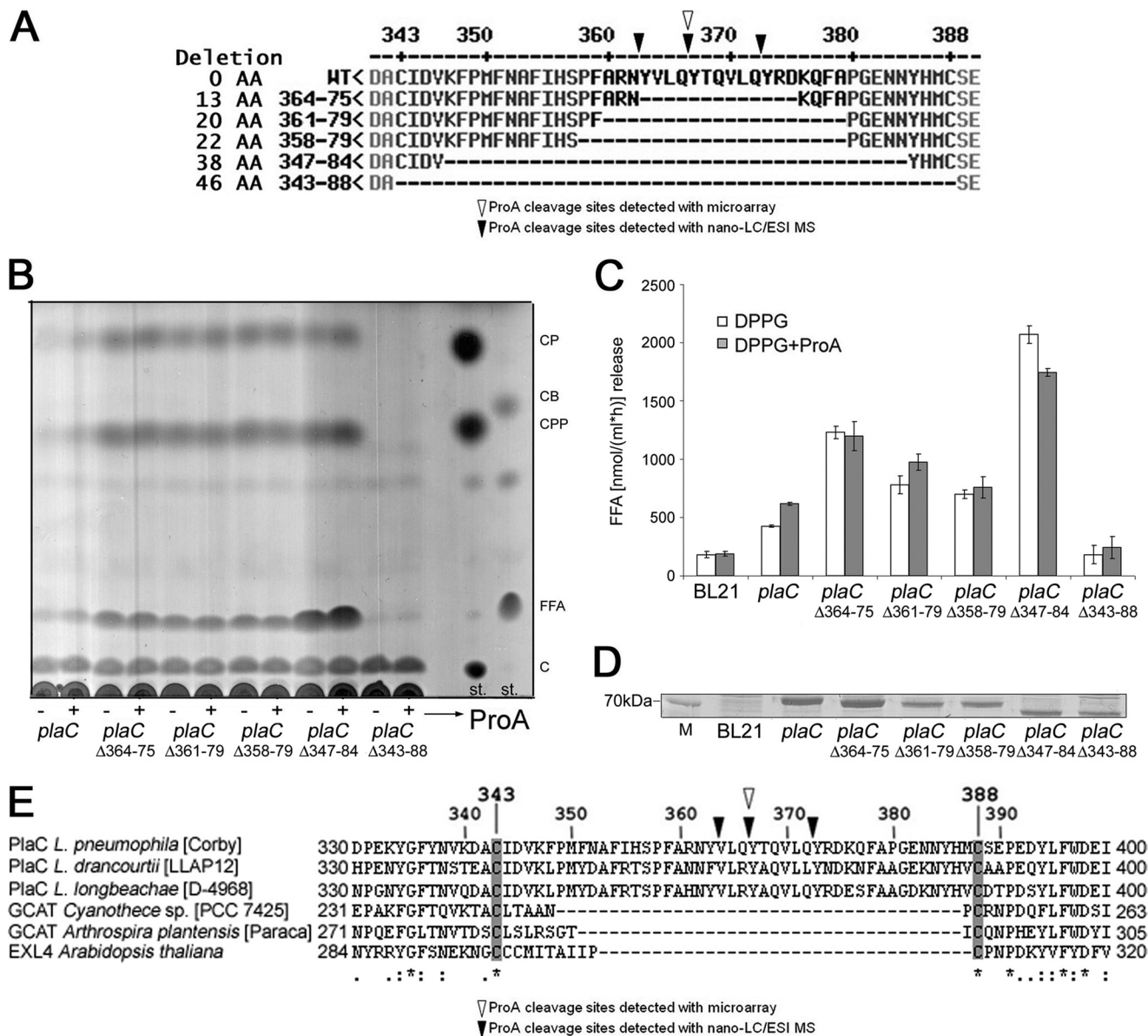
FIGURE 7. **Disulfide loop deletion independently of ProA activates PlaC-dependent PLA and GCAT activities.** An overview of the PlaC region between amino acids 341 and 390 highlighting the potential ProA cleavage sites as identified by means of the peptide cleavage microarray and nano-LC/ESI-MS as well as PlaC loop-deletion mutants is shown (*A*). Cell lysates of *E. coli* clones expressing *plaC* (BL21(pGEX *plaC* = pER32)) and/or mutants (BL21(pGEX *plaC*$_{\Delta364-75}$ = pCL77), BL21(pGEX *plaC*$_{\Delta361-79}$ = pCL78), BL21(pGEX *plaC*$_{\Delta358-79}$ = pCL79), BL21(pGEX *plaC*$_{\Delta347-84}$ = pCL85), and BL21(pGEX *plaC*$_{\Delta343-88}$ = pCL80)) were incubated with DPPG/cholesterol supplemented with Tris-HCl or the periplasmic fraction of BL21(pet28a *proA* = pCL15) for 3 h, and then lipids were extracted and analyzed for sterol ester formation by means of TLC (*B*). The *E. coli* preparations were further incubated solely with DPPG supplemented with Tris-HCl or the periplasmic fraction of BL21(pet28a *proA* = pCL15) to determine PLA/LPLA activities via quantification of FFA (*C*). The results represent the means and standard deviations of duplicate cultures and three reactions each and are representative of three independent experiments. Comparable expression of the different *plaC* constructs was shown by detection of the overexpressed 74-kDa GST-PlaC proteins in reducing SDS-PAGE and Coomassie Blue staining (*D*). The disulfide loop region (PlaC amino acids 330–400) ClustalW2 multiple alignment of different PlaC-like GDSL proteins of *Legionella* ssp., different cyanobacterial species, and *A. thaliana* extracellular lipase EXL4 highlighting the potential ProA cleavage sites is shown (*E*). *st.*, standard; *CB*, cholesterol butyrate; *C*, cholesterol; *M*, molecular weight marker; *AA*, amino acids; * indicates positions which have a single, fully conserved residue, : indicates conservation between groups of strongly similar properties, and . indicates conservation between groups of weakly similar properties.

We have now mechanistically elucidated GCAT activation of a bacterial GDSL protein by a bacterial metalloproteinase. So far, only bacterial serine proteinase-dependent activation in *A. salmonicida* SatA and eukaryotic RhoA-dependent activation for *Salmonella enterica* SseJ have been described (48, 49, 59–61).

The *L. pneumophila* zinc metalloproteinase ProA fulfils a variety of functions that may trigger bacterial pathogenicity. It degrades host extracellular matrix and immunity proteins, sup-

ports *Hartmanella vermiformis* amoebae and guinea pig infection, and is indeed found in the *L. pneumophila* phagosome (62–64). Here we showed that the enzyme also regulates lipolytic activities of the pathogen that might contribute to the observed loss of hemolytic activities in a *L. pneumophila proA* knock-out mutant (65, 66).

As shown in the case of PlaC GCAT activation, disulfide loop reduction/deletion rather than cleavage at a single site as a precondition for enzyme activation to our knowledge has not yet
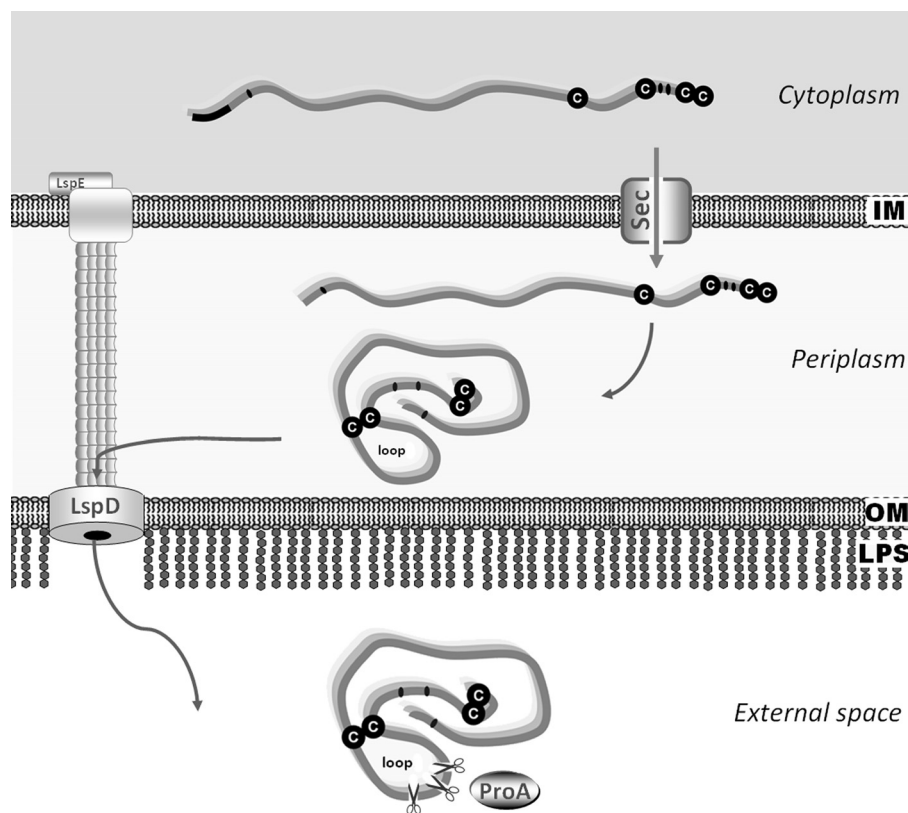
FIGURE 8. **Proposed model for ProA-dependent activation of *L. pneumophila* PlaC GCAT activity.** Within the cytoplasm, PlaC and ProA are present in their inactive preproform. After transport of PlaC and ProA through the inner bacterial membrane via the Sec system and concomitant signal peptide cleavage, ProA is present in its inactive proform. In the periplasm, a disulfide bridge(s) may be formed within PlaC, which is then still found in its GCAT-inactive proform. After subsequent export of ProA and PlaC via the Lsp secretion system, ProA acquires its mature active form via autoactivation by means of N-terminal propeptide cleavage. PlaC is then processed and thereby activated by mature ProA and subsequently shows GCAT and full PLA activity. *IM*, inner bacterial membrane; *OM*, outer bacterial membrane; *LPS*, lipopolysaccharide.

been described. There are other examples of eukaryotic and bacterial enzymes where proteolytic processing within a protein loop structure and often subsequent release of individual protein domains result in increased activity. For example, RipA, an endopeptidase of *Mycobacterium tuberculosis*, is expressed as a zymogen and activated via proteolytic processing of an exposed loop connecting the prodomain with the catalytic domain, resulting in the release of the catalytic domain (67). In other cases, such as *Pseudomonas* exotoxin A, enzyme activation is triggered by proteolytic cleavage at Arg-279 between two disulfide bridge-forming cysteines, and after a following reduction of the disulfide bridge, the activity domain is released (55, 68). Several bacterial toxins, including diphtheria toxin, Shiga toxin, cholera toxin, and *E. coli* heat-labile toxin, require activation by bacterial or host proteinases (55, 69–71). For example, the C-terminal region of Shiga toxin subunit A contains two disulfide bridge-linked cysteines forming a loop that contains the trypsin and furin proteinase recognition sequence Arg-Val-Ala-Arg (72). Cleavage at a single site results in enzymatically active disulfide bridge-linked A1 and A2 fragments (70).

Conformational changes associated with loop cleavage seem important for the regulation of enzyme activity and substrate binding. In some cases, such as RipA of *M. tuberculosis*, the loop binds tightly to the catalytic cleft, for example by means of several hydrogen bridges (67). It is possible that reduction/deletion

of the possible Cys-343/Cys-388 loop structure in PlaC may eliminate a sterically inhibiting structure and then results in an "open gate" conformation for more efficient fatty acid substrate donor and acceptor binding. Because a reduced level of PLA and full LPLA activity was observed before ProA activation (Fig. 3*C*), we speculate that the more voluminous diacylphospholipid substrates might get better access to the substrate binding or catalytic domains after loop reduction/removal and that access to the acceptor molecule might only be possible after proteolytic processing. Interestingly, similar PlaC Cys-343/Cys-388 loop protein sequences were only found in PlaC-like *Legionella* enzymes, suggesting that the protein region targeted by ProA for GCAT activation is perhaps genus-specific. However, as our own experiments suggest, the mechanism of activation by loop reduction/deletion may be more universal. A further possible target of a similar activation is *L. pneumophila* PlaA acyltransferase whose activation here was shown to be ProA-dependent (Fig. 3*E*). Importantly, the localization of potentially loop-forming cysteine residues in many other GDSL enzymes, including PlaA, is conserved as they are generally located between homology block IV and V.[3] Even more intriguing, we found several GDSL enzymes of different cya-

---

[3] C. Lang and A. Flieger, unpublished observation.

nobacteria annotated as phosphatidylcholine-sterol *O*-acyl-transferases and plant enzymes, such as the extracellular *A. thaliana* GDSL esterase/lipase EXL4, that only showed a very small protein region of about 8–10 amino acids between the potentially disulfide bridge-forming cysteines (Fig. 7*E*), suggesting that those enzymes may be active even without proteinase activation.

Here we found that the three potential catalytic amino acids of PlaC, namely Ser-37, Asp-396, and His-401, were all required for both PLA and GCAT activities. The three catalytic amino acids are conserved in the three *L. pneumophila* GDSL enzymes and are strictly conserved in other GDSL enzymes, suggesting that all enzymes of this family additionally possess acyltransferase activity. Indeed, here we showed that, in addition to PlaC, PlaA and PlaD also contribute to sterol acylation (Fig. 3). The latter two were not able to transfer palmitic acid but rather short chain fatty acids. Although the origin of the short chain fatty acid, especially propionic acid, substrates is not clear, they might be a minor constituent of the DPPG substrate used.

PlaC, PlaA, and to some extent PlaD acylate ergosterol, a typical membrane lipid of protozoa (including amoebae), fungi, and microalgae (Fig. 3, *B* and *E*) (43–45). Free living amoebae inhabiting aquatic systems are the predominant host for *Legionella* intracellular growth in natural environments (73). Ergosterol substitutes for mammalian cholesterol as a major sterol in many protozoan/amoebal cells, including *Acanthamoeba* (42, 44). Sterols are essential components of the membranes modulating membrane fluidity, signal transduction, and activity of membrane-bound enzymes (45). Therefore, acylation of sterols present not only in protozoans but also in the accidental human host provides *Legionella* with an important tool for host cell manipulation. However, specific processes that are targeted by the *L. pneumophila* GCATs remain to be established in the future.

REFERENCES

1. Fields, B. S. (1996) The molecular ecology of legionellae. *Trends Microbiol.* **4,** 286–290
2. Hilbi, H., Jarraud, S., Hartland, E., and Buchrieser, C. (2010) Update on Legionnaires' disease: pathogenesis, epidemiology, detection and control. *Mol. Microbiol.* **76,** 1–11
3. Winn, W. C., Jr., and Myerowitz, R. L. (1981) The pathology of the *Legionella* pneumonias. A review of 74 cases and the literature. *Hum. Pathol.* **12,** 401–422
4. Banerji, S., Aurass, P., and Flieger, A. (2008) The manifold phospholipases A of *Legionella pneumophila*—identification, export, regulation, and their link to bacterial virulence. *Int. J. Med. Microbiol.* **298,** 169–181
5. Lang, C., and Flieger, A. (2011) Characterisation of *Legionella pneumophila* phospholipases and their impact on host cells. *Eur. J. Cell Biol.* **90,** 903–912
6. Holk, A., Rietz, S., Zahn, M., Quader, H., and Scherer, G. F. (2002) Molecular identification of cytosolic, patatin-related phospholipases A from *Arabidopsis* with potential functions in plant signal transduction. *Plant Physiol.* **130,** 90–101
7. Banerji, S., and Flieger, A. (2004) Patatin-like proteins: a new family of lipolytic enzymes present in bacteria? *Microbiology* **150,** 522–525
8. Bender, J., Rydzewski, K., Broich, M., Schunder, E., Heuner, K., and Flieger, A. (2009) Phospholipase PlaB of *Legionella pneumophila* represents a novel lipase family: protein residues essential for lipolytic activity, substrate specificity, and hemolysis. *J. Biol. Chem.* **284,** 27185–27194
9. Buckley, J. T., Halasa, L. N., Lund, K. D., and MacIntyre, S. (1981) Purification and some properties of the hemolytic toxin aerolysin. *Can. J. Biochem.* **59,** 430–435
10. Cazalet, C., Rusniok, C., Brüggemann, H., Zidane, N., Magnier, A., Ma, L., Tichit, M., Jarraud, S., Bouchier, C., Vandenesch, F., Kunst, F., Etienne, J., Glaser, P., and Buchrieser, C. (2004) Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. *Nat. Genet.* **36,** 1165–1173
11. Chien, M., Morozova, I., Shi, S., Sheng, H., Chen, J., Gomez, S. M., Asamani, G., Hill, K., Nuara, J., Feder, M., Rineer, J., Greenberg, J. J., Steshenko, V., Park, S. H., Zhao, B., Teplitskaya, E., Edwards, J. R., Pampou, S., Georghiou, A., Chou, I. C., Iannuccilli, W., Ulz, M. E., Kim, D. H., Geringer-Sameth, A., Goldsberry, C., Morozov, P., Fischer, S. G., Segal, G., Qu, X., Rzhetsky, A., Zhang, P., Cayanis, E., De Jong, P. J., Ju, J., Kalachikov, S., Shuman, H. A., and Russo, J. J. (2004) The genomic sequence of the accidental pathogen *Legionella pneumophila*. *Science* **305,** 1966–1968
12. D'Auria, G., Jiménez-Hernández, N., Peris-Bondia, F., Moya, A., and Latorre, A. (2010) *Legionella pneumophila* pangenome reveals strain-specific virulence factors. *BMC Genomics* **11,** 181
13. Glöckner, G., Albert-Weissenberger, C., Weinmann, E., Jacobi, S., Schunder, E., Steinert, M., Hacker, J., and Heuner, K. (2008) Identification and characterization of a new conjugation/type IVA secretion system (trb/tra) of *Legionella pneumophila* Corby localized on two mobile genomic islands. *Int. J. Med. Microbiol.* **298,** 411–428
14. Hilton, S., and Buckley, J. T. (1991) Action of a microbial lipase/acyltransferase on phospholipid monolayers. *Biochemistry* **30,** 6070–6074
15. Hilton, S., and Buckley, J. T. (1991) Studies on the reaction mechanism of a microbial lipase/acyltransferase using chemical modification and site-directed mutagenesis. *J. Biol. Chem.* **266,** 997–1000
16. Brick, D. J., Brumlik, M. J., Buckley, J. T., Cao, J. X., Davies, P. C., Misra, S., Tranbarger, T. J., and Upton, C. (1995) A new family of lipolytic plant enzymes with members in rice, *Arabidopsis* and maize. *FEBS Lett.* **377,** 475–480
17. Upton, C., and Buckley, J. T. (1995) A new family of lipolytic enzymes? *Trends Biochem. Sci.* **20,** 178–179
18. Mølgaard, A., Kauppinen, S., and Larsen, S. (2000) Rhamnogalacturonan acetylesterase elucidates the structure and function of a new family of hydrolases. *Structure* **8,** 373–383
19. Arpigny, J. L., and Jaeger, K. E. (1999) Bacterial lipolytic enzymes: classification and properties. *Biochem. J.* **343,** 177–183
20. Flieger, A., Gong, S., Faigle, M., Stevanovic, S., Cianciotto, N. P., and Neumeister, B. (2001) Novel lysophospholipase A secreted by *Legionella pneumophila*. *J. Bacteriol.* **183,** 2121–2124
21. Flieger, A., Neumeister, B., and Cianciotto, N. P. (2002) Characterization of the gene encoding the major secreted lysophospholipase A of *Legionella pneumophila* and its role in detoxification of lysophosphatidylcholine. *Infect. Immun.* **70,** 6094–6106
22. DebRoy, S., Dao, J., Söderberg, M., Rossier, O., and Cianciotto, N. P. (2006) *Legionella pneumophila* type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 19146–19151
23. Rossier, O., Starkenburg, S. R., and Cianciotto, N. P. (2004) *Legionella pneumophila* type II protein secretion promotes virulence in the A/J mouse model of Legionnaires' disease pneumonia. *Infect. Immun.* **72,** 310–321
24. Creasey, E. A., and Isberg, R. R. (2012) The protein SdhA maintains the integrity of the *Legionella*-containing vacuole. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 3481–3486
25. Banerji, S., Bewersdorff, M., Hermes, B., Cianciotto, N. P., and Flieger, A. (2005) Characterization of the major secreted zinc metalloprotease-dependent glycerophospholipid:cholesterol acyltransferase, PlaC, of *Legion-*

ella pneumophila. Infect. Immun. **73,** 2899–2909

26. Hales, L. M., and Shuman, H. A. (1999) *Legionella pneumophila* contains a type II general secretion pathway required for growth in amoebae as well as for secretion of the Msp protease. *Infect. Immun.* **67,** 3662–3666

27. Jepras, R. I., Fitzgeorge, R. B., and Baskerville, A. (1985) A comparison of virulence of two strains of *Legionella pneumophila* based on experimental aerosol infection of guinea-pigs. *J. Hyg.* **95,** 29–38

28. Bertani, G. (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli. J. Bacteriol.* **62,** 293–300

29. Edelstein, P. H. (1981) Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *J. Clin. Microbiol.* **14,** 298–303

30. Moffat, J. F., Black, W. J., and Tompkins, L. S. (1994) Further molecular characterization of the cloned *Legionella pneumophila* zinc metalloprotease. *Infect. Immun.* **62,** 751–753

31. Stone, B. J., and Kwaik, Y. A. (1999) Natural competence for DNA transformation by *Legionella pneumophila* and its association with expression of type IV pili. *J. Bacteriol.* **181,** 1395–1402

32. Flieger, A., Rydzewski, K., Banerji, S., Broich, M., and Heuner, K. (2004) Cloning and characterization of the gene encoding the major cell-associated phospholipase A of *Legionella pneumophila*, plaB, exhibiting hemolytic activity. *Infect. Immun.* **72,** 2648–2658

33. Broich, M., Rydzewski, K., McNealy, T. L., Marre, R., and Flieger, A. (2006) The global regulatory proteins LetA and RpoS control phospholipase A, lysophospholipase A, acyltransferase, and other hydrolytic activities of *Legionella pneumophila* JR32. *J. Bacteriol.* **188,** 1218–1226

34. Conlan, J. W., Baskerville, A., and Ashworth, L. A. (1986) Separation of *Legionella pneumophila* proteases and purification of a protease which produces lesions like those of Legionnaires' disease in guinea pig lung. *J. Gen. Microbiol.* **132,** 1565–1574

35. Prestidge, L., Gage, V., and Spizizen, J. (1971) Protease activities during the course of sporulation on *Bacillus subtilis. J. Bacteriol.* **107,** 815–823

36. Thorpe, T. C., and Miller, R. D. (1981) Extracellular enzymes of *Legionella pneumophila. Infect. Immun.* **33,** 632–635

37. Neu, H. C., and Heppel, L. A. (1965) The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J. Biol. Chem.* **240,** 3685–3692

38. Flieger, A., Gongab, S., Faigle, M., Mayer, H. A., Kehrer, U., Mussotter, J., Bartmann, P., and Neumeister, B. (2000) Phospholipase A secreted by *Legionella pneumophila* destroys alveolar surfactant phospholipids. *FEMS Microbiol. Lett.* **188,** 129–133

39. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685

40. Ahrends, R., Pieper, S., Neumann, B., Scheler, C., and Linscheid, M. W. (2009) Metal-coded affinity tag labeling: a demonstration of analytical robustness and suitability for biological applications. *Anal. Chem.* **81,** 2176–2184

41. Black, W. J., Quinn, F. D., and Tompkins, L. S. (1990) *Legionella pneumophila* zinc metalloprotease is structurally and functionally homologous to *Pseudomonas aeruginosa* elastase. *J. Bacteriol.* **172,** 2608–2613

42. Korn, E. D., and Wright, P. L. (1973) Macromolecular composition of an amoeba plasma membrane. *J. Biol. Chem.* **248,** 439–447

43. Raederstorff, D., and Rohmer, M. (1985) Sterol biosynthesis de nova via cycloartenol by the soil amoeba *Acanthamoeba polyphaga. Biochem. J.* **231,** 609–615

44. Smith, F. R., and Korn, E. D. (1968) 7-Dehydrostigmasterol and ergosterol: the major sterols of an amoeba. *J. Lipid Res.* **9,** 405–408

45. Volkman, J. K. (2003) Sterols in microorganisms. *Appl. Microbiol. Biotechnol.* **60,** 495–506

46. Akoh, C. C., Lee, G. C., Liaw, Y. C., Huang, T. H., and Shaw, J. F. (2004) GDSL family of serine esterases/lipases. *Prog. Lipid Res.* **43,** 534–552

47. Brumlik, M. J., and Buckley, J. T. (1996) Identification of the catalytic triad of the lipase/acyltransferase from *Aeromonas hydrophila. J. Bacteriol.* **178,** 2060–2064

48. Eggset, G., Bjornsdottir, R., McQueen Leifson, R., and Arnesen, J. A. (1994) Extracellular glycerophospholipid:cholesterol acyltransferase from *Aeromonas salmonicida*: activation by serine protease. *J. Fish Dis.* **17,** 17–29

49. Hilton, S., McCubbin, W. D., Kay, C. M., and Buckley, J. T. (1990) Purification and spectral study of a microbial fatty acyltransferase: activation by limited proteolysis. *Biochemistry* **29,** 9072–9078

50. Ferrè, F., and Clote, P. (2005) DiANNA: a web server for disulfide connectivity prediction. *Nucleic Acids Res.* **33,** W230–W232

51. Choe, S., Bennett, M. J., Fujii, G., Curmi, P. M., Kantardjieff, K. A., Collier, R. J., and Eisenberg, D. (1992) The crystal structure of diphtheria toxin. *Nature* **357,** 216–222

52. Downing, M. R., Butkowski, R. J., Clark, M. M., and Mann, K. G. (1975) Human prothrombin activation. *J. Biol. Chem.* **250,** 8897–8906

53. Duckworth, W. C., Stentz, F. B., Heinemann, M., and Kitabchi, A. E. (1979) Initial site of insulin cleavage by insulin protease. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 635–639

54. Fasman, G. D., Foster, R. J., and Beychok, S. (1966) The conformational transition associated with the activation of chymotrypsinogen to chymotrypsin. *J. Mol. Biol.* **19,** 240–253

55. Gordon, V. M., and Leppla, S. H. (1994) Proteolytic activation of bacterial toxins: role of bacterial and host cell proteases. *Infect. Immun.* **62,** 333–340

56. Geiger, O. (2010) in *Handbook of Hydrocarbon and Lipid Microbiology*, pp. 3195–3202, Springer, New York

57. Goldfine, H. (1982) Lipids of prokaryotes—structure and distribution. *Curr. Top. Membr. Transp.* **17,** 1–43

58. Sturt, H. F., Summons, R. E., Smith, K., Elvert, M., and Hinrichs, K. U. (2004) Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry—new biomarkers for biogeochemistry and microbial ecology. *Rapid Commun. Mass Spectrom.* **18,** 617–628

59. Lossi, N. S., Rolhion, N., Magee, A. I., Boyle, C., and Holden, D. W. (2008) The *Salmonella* SPI-2 effector SseJ exhibits eukaryotic activator-dependent phospholipase A and glycerophospholipid:cholesterol acyltransferase activity. *Microbiology* **154,** 2680–2688

60. Nawabi, P., Catron, D. M., and Haldar, K. (2008) Esterification of cholesterol by a type III secretion effector during intracellular *Salmonella* infection. *Mol. Microbiol.* **68,** 173–185

61. Christen, M., Coye, L. H., Hontz, J. S., LaRock, D. L., Pfuetzner, R. A., Megha, and Miller, S. I. (2009) Activation of a bacterial virulence protein by the GTPase RhoA. *Sci. Signal.* **2,** ra71

62. Moffat, J. F., Edelstein, P. H., Regula, D. P., Jr., Cirillo, J. D., and Tompkins, L. S. (1994) Effects of an isogenic Zn-metalloprotease-deficient mutant of *Legionella pneumophila* in a guinea-pig pneumonia model. *Mol. Microbiol.* **12,** 693–705

63. Rossier, O., Dao, J., and Cianciotto, N. P. (2008) The type II secretion system of *Legionella pneumophila* elaborates two aminopeptidases, as well as a metalloprotease that contributes to differential infection among protozoan hosts. *Appl. Environ. Microbiol.* **74,** 753–761

64. Okamoto, T., Akuta, T., Tamura, F., van Der Vliet, A., and Akaike, T. (2004) Molecular mechanism for activation and regulation of matrix metalloproteinases during bacterial infections and respiratory inflammation. *Biol. Chem.* **385,** 997–1006

65. Quinn, F. D., and Tompkins, L. S. (1989) Analysis of a cloned sequence of *Legionella pneumophila* encoding a 38 kD metalloprotease possessing haemolytic and cytotoxic activities. *Mol. Microbiol.* **3,** 797–805

66. Keen, M. G., and Hoffman, P. S. (1989) Characterization of a *Legionella pneumophila* extracellular protease exhibiting hemolytic and cytotoxic activities. *Infect. Immun.* **57,** 732–738

67. Ruggiero, A., Marasco, D., Squeglia, F., Soldini, S., Pedone, E., Pedone, C., and Berisio, R. (2010) Structure and functional regulation of RipA, a mycobacterial enzyme essential for daughter cell separation. *Structure* **18,** 1184–1190

68. Ogata, M., Fryling, C. M., Pastan, I., and FitzGerald, D. J. (1992) Cell-mediated cleavage of *Pseudomonas* exotoxin between Arg[279] and Gly[280] generates the enzymatically active fragment which translocates to the cytosol. *J. Biol. Chem.* **267,** 25396–25401

69. Giannelli, V., Fontana, M. R., Giuliani, M. M., Guangcai, D., Rappuoli, R., and Pizza, M. (1997) Protease susceptibility and toxicity of heat-labile enterotoxins with a mutation in the active site or in the protease-sensitive loop. *Infect. Immun.* **65,** 331–334

70. Kurmanova, A., Llorente, A., Polesskaya, A., Garred, O., Olsnes, S., Kozlov, J., and Sandvig, K. (2007) Structural requirements for furin-induced cleavage and activation of Shiga toxin. *Biochem. Biophys. Res. Commun.* **357,** 144–149

71. Tsuneoka, M., Nakayama, K., Hatsuzawa, K., Komada, M., Kitamura, N., and Mekada, E. (1993) Evidence for involvement of furin in cleavage and activation of diphtheria toxin. *J. Biol. Chem.* **268,** 26461–26465

72. Garred, O., van Deurs, B., and Sandvig, K. (1995) Furin-induced cleavage and activation of Shiga toxin. *J. Biol. Chem.* **270,** 10817–10821

73. Winiecka-Krusnell, J., and Linder, E. (1999) Free-living amoebae protecting *Legionella* in water: the tip of an iceberg? *Scan. J. Infect. Dis.* **31,** 383–385