3.2 Methods

3.2.1 Growth conditions of bacterial and eukaryotic strains

3.2.1.1 Bacterial strains and growth conditions

L. pneumophila sg1 strain 130b was used for mutagenesis of the *plaA*, *plaC*, *plaD*, *patA*, *unk1*, *lvrE*, and *aas* genes and later served as the corresponding wild type control. The *plaA* mutant, a 130b derivative, is defective in the major secreted LPLA activity (Table 3.23) (75). The *lspDE* and *proA* mutants, two further derivatives of 130b, are defective in the type II secretion system or contain a knockout of the zinc metalloprotease gene, respectively (Table 3.23) (131, 163). Furthermore, *L. pneumophila* sg1 strain Corby was used for mutagenesis of the *plaB*, *plaA*, *plaC*, and *plaD* genes and later served as the corresponding wild type control (76). Finally, the *L. pneumophila* sg1 strain Philadelphia-1 was used for mutagenesis of the *lvrE* gene and later served as the corresponding wild type control.

For long-term conservation, *L. pneumophila* and *E. coli* were stored in glycerol stocks, i.e. in broth and glycerol in a ratio of 1:1 (v/v), at -80 °C. *L. pneumophila* mother plates from wild type or mutants were generated by plating an aliquot from a glycerol stock on buffered charcoal-yeast extract (BCYE) agar (with or without antibiotics) and incubating it for 2 days at 37 °C (61). These mother plates were kept for 4-6 weeks at 4°C and used for routinely growing *L. pneumophila* on BCYE for 1-3 days at 37°C (daughter plates). In order to examine extracellular growth, *L. pneumophila* cell material from a daughter plate was cultured in buffered yeast extract (BYE) broth at 37°C with shaking at 350 rpm. Bacterial growth was checked by determining the optical density of the culture at wavelength 660 nm (OD_{660}; Beckman spectrophotometer DU520, Beckman Coulter) after inoculation to an OD_{660} of 0.2 to 0.4. When appropriate, media were supplemented with antibiotics at final concentrations suitable for *L. pneumophila* (Table 3.8).

Escherichia coli strain DH5α, the host for new recombinant plasmids, was grown from a glycerol stock in Luria-Bertani (LB) agar for 1 day to generate mother plates. These mother plates were kept for 6-8 weeks at 4 °C and used for generating fresh daughter plates. In LB broth, *E. coli* was grown to an OD_{660} of 1.0-1.1 and IPTG was added to a final concentration of 1mM in order to induce a P_{tac} or P_{lac} promoter on the respective vector and the bacterial

growth was continued to an OD_{660} of 2.0-2.1. Media were supplemented with antibiotics at final concentrations suitable for *E. coli* (Table 3.8).

3.2.1.2 Eucaryotic cells and growth conditions

Three host models were used in order to monitor intracellular infection by *L. pneumophila* wild type strains and corresponding mutants: *A. castellanii* amoebae, a natural host, U937 macrophages, and A549 epithelial cells, both representing opportunistic hosts.

All eukaryotic cells were kept as glycerol stocks (approximately 10^6 cells in RPMI +10 % Glycerol+10 % DMSO) at -80 °C and were rapidly thawed at 37 °C and centrifuged for 2 min at 170 × g. Pellets were washed in the appropriate culture broth (PYG for *A. castellanii* and RMPI + 10 % FCS for U937 and A549 cells) (Table 3.7) and centrifuged for 10 min at 170 × g. Then, cells were suspended in 10 ml of the appropriate culture broth and transferred to a culture flask and grown for 3-4 days at 37 °C and 5 % CO_2. Subsequently, cells were routinely passaged in intervals of 3-4 days in a ratio of 1:10 (*v/v*) for a maximum of 30 passages and used for the infection experiments.

3.2.2 DNA techniques

DNA agarose gel electrophoresis was performed with 1% agarose (0.8 % for subsequent Southern blot) for approximately 45-70 min at 90-120 V and then stained with ethidium bromide. For restriction of vectors and constructs for cloning, approximately 50 ng-150 ng of DNA was restricted using appropriate endonucleases according to the manufacturer's instructions in a volume of 50 µl for 3 -5 h (over night only for analytical purposes).

Please refer to the "Molecular cloning: A laboratory manual" from Sambrook and coworkers (1989) for (a more detailed) description of the standard DNA techniques which were employed (e.g. agarose gel electrophoresis, restriction digestion, ethanol precipitation) and which are not (or not in detail) described here.

3.2.2.1 PCR

a) PCR amplification from purified DNA

E. coli DH5α was employed for propagation of recombinant plasmid DNA. First, the desired fragment was amplified from the respective *L. pneumophila* genome by PCR using PlatinumTaq Highfidelity and the appropriate primer pairs (Tables 3.12-3.17) according to

the manufacturer's instructions and cloned into the vector pGEMTez. In some cases, where the amplified product was directly used for blunt-end ligation (e.g. ligation into *Sma*I linearized pMMB2002) the amplification was carried out with *Pfu* polymerase according to the manufacturer's instructions. The following protocol and
PCR program were used for amplification:

Components	Volumes
DNA	1 µl
Buffer	5 µl
Mg SO_4 (50 mM)	2 µl
dNTP-Mix (each dNTP 10 mM)	1 µl
Primer 1 (50 mM)	1 µl
Primer 2 (50 mM)	1 µl
PlatinumTaq	0.5 µl
H_2O_{bidest} ad	50 µl

Reaction	Number of cycles	Time	Temperature
Denaturation	1	2 min	95°C
Denaturation		30 sec	95°C
Annealing	30	30 sec	52°C-64°C
Elongation		4 min	68°C (PlatiTaq) 72°C (Pfu)
Final elongation	1	7 min	72°C

b) Colony PCR

Colony PCR was used as a rapid method to check for clones carrying a desired gene or for the insertion of a resistance gene (Km^R and Gm^R) cassette in *L. pneumophila* mutants. A single colony grown on agar was transferred into a PCR tube and resuspended in 21 µl sterile H_2O_{bidest} and 2.5 µl PCR buffer *Taq* (includes $MgSO_4$) and lysed by boiling at 95 °C for 10 min. Then the other components essential for a PCR were added (10 mM each dNTP, 50 µM each primer, 5 U/µl Taq polymerase) in a final volume of 25.25 µl. The following PCR program was used for Colony PCR:

Reaction	Number of cycles	Time	Temperature
Denaturation		60 sec	95°C
Annealing	30	60 sec	56°C
Elongation		60 sec	72°C
Final elongation	1	7 min	72°C

c) Reverse-transcriptase (RT)-PCR

For the analysis of gene expression, m-RNA was amplified by RT-PCR using the Quiagen OneStep RT-PCR kit. Control reactions to check for the presence of mRNA were performed using the primer pairs for the *L. pneumophila* or *E. coli* gyrase gene (Table 3.17). First, the RNA is transcribed into cDNA at 50 °C by the reverse transcriptase which is then destroyed by incubation at 95 °C which also activates the HotStarTaq DNA polymerase of the enzyme mix. Then a conventional PCR is performed. To check for DNA contamination, control tubes are placed into the cycler during step 2 (95°C). The following protocol, program, and primers were used:

Components	Volumes	Final concentrations
RNase-free H_2O	x µl	-
5x RT-PCR buffer	5 µl	1×
dNTP-Mix (each dNTP 10 mM)	1 µl	400 µM of each dNTP
Primer 1	0.3 µl	0.6 µM
Primer 2	0.3 µl	0.6 µM
Enzyme mix	1 µl	-
Template RNA	x µl	1 pg – 2 µg/reaction
Final volume	25 µl	

Reaction	Number of cycles	Time	Temperature
Reverse transkription	1	30 min	50°C
Destruction of reverse tanscriptase	1	15 min	95°C
Denaturation		1 min	94°C

Reaction	Number of cycles	Time	Temperature
Annealing	30	1 min	56°C – 62°C
Elongation		1 min	72°C
Final elongation	1	10 min	72°C

Gene	RT-PCR primer pairs	Annealing temperature	Fragment size [bp]
plaC	gdsl2_s2_f + gdsl2_s2_r	61.5/ 62°C	461
plaD	gdsl3_s4_f + gdsl3_s3_r	62°C	435
unk1	unk1_c1_f+unk1_d1_r	56°C	557
lvrE	lvrE_e1_f+lvrE_f1_r	56°C	556
L. pneumophila gyrase	GyrRTb-f+GyrRTb-r	61.5°C	122
	RTgyr_a1_f+RTgyr_b1_r	56°C	469
E. coli gyrase	K12gyr_a1_f+K12gyr_b1_r	64°C	391

d) DNA sequencing

Both strands of plasmid DNA were sequenced by using the ABI BigDye 3.1 Terminator Premix and an automated DNA sequencer at the sequencing facility of the Robert Koch-Institut. The following protocol and program for PCR were used:

Components	Volumes	Final concentration
Template DNA	x µl	120-250 ng
Primer	0.5 µl	10 pmol
BigDye 3.1 premix	1 µl	
5x Puffer	2 µl	
H_2O_{bidest}	x µl	
Final volume	10 µl	

Reaction	Number of cycles	Time	Temperature
Denaturation	1	1.5 min	95°C
Denaturation	25	30 sec	95°C
Annealing		15 sec	56°C
Elonation		4 min	60°C

The electrophoretic analysis of the PCR fragments was performed by the staff of the sequencing facility of the Robert Koch-Institut.

e) PCR probes for Southern hybridization

To generate Dioxygenin (DIG) labelled PCR probes for Southern hybridization, the following primers, protocol, and program for PCR were used:

a) Primers

Gene	RT-PCR primer pairs	Amplification product (bp)
plaC	gdsl2_s2_f + gdsl2_s2_r	461
plaD	gdsl3_s4_f + gdsl3_s3_r	435
unk1	unk1_c1_f+unk1_d1_r	557
lvrE	lvrE_e1_f+lvrE_f1_r	556
patA	patA_a1_f+patA_b1_r	2193
aas	aas_a1_f+aas_b1_r	2418
Km^R	Kan_a1_f/b1_r	978
Gm^R	lipBGmR_f/_r	1024

b) Protocol

Components	Volumes	Stock concentrations
Taq PCR buffer	5 µl	10× (including $MgSO_4$)
dNTP mix	1 µl	10 mM each dNTP
PCR primer 1	1 µl	50 µM
PCR primer 2	1 µl	50 µM
DIG labelling	5 µl	10×
Template DNA	x µl	20-50 ng
Taq polymerase	0.5 µl	5 U/µl
sterile H_2O	x µl	
Final volume	50 µl	

c) PCR program

Reaction	Number of cycles	Time		Temperature
Denaturation	1	5	min	95°C
Denaturation		30	sec	95°C
Annealing	30	30	sec	58°C/62°C
Elongation		2	min	72°C
Final elongation	1	7	min	72°C

3.2.2.2 Nucleic acid isolation

a) Isolation of plasmid DNA

Plasmid DNA was isolated from liquid cultures of *E. coli* clones carrying a vector with the desired insert by alkaline lysis using either the *QIAprep Miniprep Kit* from Qiagen or the *Quantum Prep Plasmid Midiprep Kit* from Bio-Rad according to the manufacturers' instructions (Table 3.5). The miniprep kit utilized 2 ml of mid logarithmic *E. coli* cultures and yielded a maximum amount of 1 µg plasmid DNA in 50 µl which was subsequently used for subcloning, sequencing, and PCR. The midiprep kit needed 40 ml of liquid culture and yielded a maximum amount of 100 µg which was eluted into 600 µl of elution buffer and was subsequently used for mutagenesis

b) Isolation of genomic DNA

Chromosomal DNA from *L. pneumophila* wild type and corresponding mutant strains was obtained by means of the E.Z.N.A. bacterial DNA kit from Peqlab according to the manufacturer's instructions (5 min lysozyme incubation, 1 h proteinase K digestion). Finally, the DNA was dissolved in 2× 50 µl of elution buffer and either directly used for Southern hybridization or was tenfold diluted for use in PCR.

c) Isolation of RNA

Bacteria grown in BYE broth to a desired optical density (OD_{660}) were diluted to an OD_{660}=0.3 with BYE broth in order to adjust comparable bacterial numbers. An aliquot of 1 ml bacteria were pelleted by centrifugation for 10 min at 5000 × g and 4 °C and pellets were stored at -20 °C until RNA was isolated. RNA was isolated using the RNeasy mini kit (Qiagen, Hilden Germany) according to the manufacturer's instructions (Table 3.5). A possible DNA

contamination was removed with RQ1 RNase-Free DNase (Promega). Briefly, RNA (500 ng-2 µg) was mixed with 1 µl 10 × DNase buffer (Promega), 1 µl RQ1 DNase (Promega), and 1 µl RNase inhibitor (Sigma) and incubated for 15 min at 37 °C. Subsequently, 1 µl STOP buffer (Promega) was added to the mixture and incubated for 15 min at 65 °C to inactivate DNase.

3.2.2.3 Gene cloning

Currently, the genomes of strains 130b and Corby have not been sequenced. Therefore, the primers used for the amplification of genes from these strains were all based on the sequence found in the *L. pneumophila* Philadelphia-1 database (38) (http://genome3.cpmc.columbia.edu/~legion). First, the desired fragment was amplified from the respective *L. pneumophila* genomic DNA by PCR using PlatinumTaq Highfidelity and the appropriate primer pairs (Tables 3.12-3.17) according to the manufacturer's instructions and cloned into the vector pGEMTez (Table 3.18). pGEMTez is a linearized vector with a polyT tail which is complementary to the polyA end of PCR products amplified with Taq polymerase. However, this vector which has only an Amp^R gene cassette is not suitable for *L. pneumophila* which is resistant to ampicillin. Therefore, the genes were subcloned into vectors pMMB2002 and/or pBCKS which have a Cm^R gene cassette. In some cases, genes were amplified with *Pfu* polymerase which generates blunt-ends and were directly ligated into *Sma*I linearized pMMB2002. For subcloning, the vector, carrying the gene of interest was digested with (two) suitable restriction enzymes for 3-5 h at 16°C or 37 °C (depending on the enzymes) and the fragment was separated by gel electrophoresis and purified from the gel by means of the QIAquick Gel Extraction Kit from Quiagen according to the manufacturer's instructions (Table 3.5). Ligation was performed over night at 16°C with DNA ligase from the bacteriophage T4 following the manufacturer's instructions in a volume which did not exceed 30 µl (Table 3.11). For sticky-end ligation, a vector:insert ratio of 1:3 was generally chosen, while for blunt-end ligation the ratio was generally 1:5.

Typical example for a ligation reaction:

Components	Volumes
Buffer (10×)	1 µl
Insert (~30 ng/µl)	6 µl
Vector (~30 ng/µl)	2 µl
T4 DNA ligase	1 µl
Total volume	10 µl

3.2.2.4 Electroporation

Foreign DNA was introduced into bacterial strains by electroporation by means of a Cell-Porator® from Life-Technologies (Paisley, Scotland) according to the manufacturer's specifications. Electroporation of *E. coli* (or *L. pneumophila*) was carried out using 200 DC Volts (400 DC Volts), 4 kΩ (4 kΩ), and 330 µF (330 µF). For electroporation, bacteria needed to be electrocompetent. *L. pneumophila* strains 130b, Corby, and Philadelphia-1 can be transformed by electropration after washing in 10 % glycerol. Therefore, bacterial cells from 2 day old BCYE agar plates were washed twice with ice-cold 10 % glycerol and resuspended in high density in ice-cold 10 % glycerol and were stored at -80 °C until electroporation. For the generation of electrocompetent *E. coli*, 500 ml SOB broth was inoculated with DH5α to an OD (600 nm) ≤ 0.1 and bacteria were grown to an OD (600 nm) of 0.8 and pelleted by centrifugation at 4°C with 5000 × g for 10 min. Bacteria were washed twice with 500 ml of ice-cold 10 % glycerol, resuspended in 2 ml of ice-cold 10 % glycerol and stored in aliquots at -80 °C until electroporation. After electroporation, bacteria were resuspended in 2 ml BYE or SOC broth for *L. pneumophila* and *E. coli*, respectively and grown for 3-5 h (*Legionella*) or 1 h (*E. coli*) before being plated-out on agar plates containing the suitable antibiotic.

3.2.2.5 Southern hybridization

Depending on the gene of interest, an aliquot of the genomic DNA (20-30 µl ~3-4.5 µg) was digested over night with different restriction enzymes listed in Table 3.28 Another aliquot was digested with AvaI which has a restriction site in the Km^R but not in the studied genes. The digested DNA was then subjected to electrophoresis. The gel was then depurinated by incubation for 5 min in depurination buffer, followed by 2 × 15 min incubation in denaturation buffer and 2 × 15 min incubation in neutralization buffer (Table 3.6.) The incubations were performed at 25 °C and the gel was washed in H_2O_{bidest} before changing the buffers. Finally the gel was equilibrated in 10 × SSC for 10 min. Subsequently, the DNA fragments were transferred to a nylon membrane by means of the Bio-Rad Vaccum Blotter following the manufacturer's instruction and were bound to the membrane by autocrosslinking (2 times).

Table 3.27 Overview of enzymes used for Southern hybridization

Gene	Restriction enzyme	Primers
Km^R	*Ava*I	kan_a1_f/b1_r
Gm^R	*Ava*I	lipB_GmR_f/_r
plaC	*Ava*II	gdsl2_a1_f/b1_r
plaD	*Mfe*I	gdsl3_c1_f/d1_r
patA	*Kpn*I	patA_a1f/b1_r
unk1	*EcoR*V	unk1_c1_f/d1_r
aas	*Acc*I	aas_a1_f/b1_r
lvrE	*Sty*I	lvrE_e1_f/f1_r

The DNA probes (see 3.2.2.1 e), generated with specific primers which are listed in Table 3.27 were used as a gene-specific probe. Hybridization with the probes was performed at 42°C overnight. Membranes were washed 2 × 5 min at room temperature in low stringency buffer (2 × SSC, 0.1 % SDS) followed by 15 min at 65°C in high stringency buffer (0.1 × SSC, 0.1 % SDS). Detection was carried out by means of CSPD, the chemiluminescent substrate for alkaline phosphatase, according to the manufacturer´s instructions (Table 3.3).

3.2.2.6 Construction of *L. pneumophila* mutants and complemented clones

Since *L. pneumophila* expresses type IV pili when grown at 30 °C which facilitate the uptake of DNA, this principle of natural transformation followed by allelic exchange was exploited to generate *L. pneumophila* mutants. As a first step, constructs were generated which contained the gene of interest interrupted by a Km^R or Gm^R gene cassette. For this purpose, the respective gene was first amplified from the *L. pneumophila* genome and cloned into pGEMTez (for *plaC*, patA, *lvrE*, *aas*) and subcloned into pBCKS (for *unk1* and *plaD*). Then, Km^R or Gm^R was amplified from the vector pET-28b or pVA15-1 (=pGEMTez+*lipB*::Gm^R) (kindly provided by N. Cianciotto) (10), respectively, using primers kan-a1_f/kan-b1_r or lipB_GmR_f/lipB_GmR_r (Table 3.19) and introduced into the respective gene in the vector. Due to lack of appropriate restriction sites for introducing the Km^R gene cassette into *unk1* and *lvrE*, the site-directed mutagenesis kit from Stratagene was employed following the manufacturer's instructions (Table 3.5). Briefly, a primer pair was generated which allowed

the amplification of the Km^R gene from pET-28b, but with additional flanking regions which were complementary to the gene of interest. The generated PCR product then served as a primer for the subsequent amplification of the gene of interest, resulting in a final construct which carried the gene of interest interrupted by Km^R at the desired position.

a) Protocol for amplification of Km^R from pET28b in a regular PCR reaction (annealing at 67 °C for both primer pairs).

Components	Volumes	
pET28b	1	µl
Buffer, (including Mg^{2+})	5	µl
dNTP-Mix (each dNTP 10 mM)	1	µl
unk1_m_f or lvrE_m_f (50 mM)	1	µl
unk1_m_f or lvrE_m_f (50 mM)	1	µl
Pfu	0.5	µl
H_2O_{bidest}	40.5	µl

b) Protocol for site-directed mutagenesis

Components	Volumes	
pBCKS+*unk1*/ pGEMTez+*lvrE*	2/6	µl
Buffer for site-directed mutagenesis	5	µl
dNTP-Mix (each dNTP 10 mM)	1	µl
PCR fragment from previous PCR	3 or 6	µl
Pfu Turbo DNA polymerase	1	µl
H_2O_{bidest} ad	50	µl

In summary, the following constructs were obtained: pBH4 (*plaD*::Km^R), pBH5 (*plaC*::Km^R), pPA5 (*patA*::Km^R), pSB10 (Δ*lvrE*::Km^R), pSB11 (*unk1*::Km^R), pSB16 (*aas*::Km^R), and pSB9 (*aas*::Gm^R). About 5-10 µg of plasmid DNA in a maximum volume of 200 µl was incubated with 2 ml of an *L. pneumophila* culture (start OD_{660}=0.2) in a plastic tube at 30 °C and 150 rpm for 18 h (end OD_{660}= 0.8-1.4). Then the bacteria were plated on BCYE agar containing kanamycin (or gentamicin) and grown for 4-6 days until single colonies were visible. PCR and Southern blot analysis were used to examine Km^R or Gm^R Legionellae for the presence of the mutation. The *L. pneumophila* Corby *plaB* mutants as well as *trans* complemented clones

were generated by Klaus Heuner (Universität Würzburg) and Kerstin Rydzewski (Robert Koch-Institut) (76). For *trans* complementation of the *plaC* mutant, the PCR product obtained with the primers gdsl2a1 and gdsl2b1 by amplification with Pfu polymerase was cloned into pMMB2002 restricted with *Sma*I yielding pMY7. pMY7 was directly transformed into an 130b *L. pneumophila plaC* mutant by electroporation. For *trans* complementation of the *L. pneumophila* 130b *unk1* mutants, plasmid pSB14 was transformed into *L. pneumophila* 130b *unk1* mutant clones 1 and 2 by electroporation. For *trans* complementation of the *lvrE* mutants, pMMBN2002::*lvrE* (generous gift by Emmy De Buck, Leuven, Belgium) or pSB18 (pMMB2002+*lvrE*) was electroporated into the *L. pneumophila* Philadelphia-1 mutant clone 19, and *L. pneumophila* 130b mutant clones 1A, 2A, and 4C.

3.2.3 Preparations of culture supernatants and cell lysates

Culture supernatants for assessment of hydrolytic activities were obtained at the end of exponential growth (i.e. OD_{660} of 2.0-2.1) or as stated at the individual experiment by centrifugation for 5 min at 5000 × g. Cell lysates were produced by the following method (75). Bacteria from the desired growth phase were pelleted by centrifugation as described above and then lysed by addition of a 1/20 volume of the original culture volume of 10 mg of lysozyme/ml and 1 µl of Triton X-100/ml at 37°C for 30 min. After repeated passage through a 26-gauge needle, the lysates were finally resuspended in 1/4 of the original culture volume in 40 mM Tris-HCl pH 7.5 (25°C) for *E. coli* lysates (except for clones carrying the *plaB* gene, which were resuspended in the original culture volume or were diluted) or were resuspended in the original culture volume in Tris-HCl for *L. pneumophila* lysates. If appropriate, cell lysates were diluted in Tris-HCl as indicated at the individual experiment. In order to assay for 2-acylglycerophospholipid acyltransferase activity of *L. pneumophila aas* mutants and related strains, the pelleted cells were fractionated into an osmotic shockate fraction and a lysate fraction (156). Pelleted *L. pneumophila* bacteria from 2 ml of liquid culture were resuspended and incubated for 10 min with shaking at 200 rpm in 1/20 of a volume of 'osmotic shock buffer' (20 % sucrose (w/v) in TE buffer) (Table 3.6) at 25 °C. Cells were pelleted at 13000 rpm in an Eppendorf centrifuge at 4 °C, the supernatant was discarded and cells were resuspended in ¼ of the original culture volume in H_2O_{dest} and again incubated for 10 min with shaking at 200 rpm and 4 °C. The cells were again centrifuged for 10 min at 5000 × g. The supernatant containing the osmotic shockate was transferred into a new tube. The pelleted cells were resuspended in 500 µl of 40 mM Tris-HCl (pH 7.5) and lysed by the addition of 2 µl Triton X-100 and ultrasonication with a probe (Sonoplus, Bandelin, Berlin, Germany) for 1 × 15 s at cycle 4 × 10 % and power % set to

65%. Culture supernatants and cell lysates were tested either immediately for enzymatic activities or were stored overnight at 4°C.

3.2.4 Assays and detection methods

3.2.4.1 Enzymatic assay for lipolytic activities

25 µl (or 50 µl for subsequent TLC analysis) of lipids were incubated with the same volume of bacterial culture supernatant or cell lysate in a mixture containing 6.7 mM substrate [1,2-dipalmitoylphosphatidylcholine (DPPC), 1,2- dipalmitoylphosphatidylglycerol (DPPG), 1-monopalmitoyllysophosphatidylcholine (MPLPC), 1-monopalmitoyllysophosphatidylglycerol (MPLPG), or 1-monopalmitoylglycerol (1-MPG)], 3 mM NaN_3, 0.5 % (v/v) Triton X-100 and 40 mM Tris-HCl pH 7.5 (25°C). Prior to incubation, the lipid substrates were vortexed for 15 min at 37°C and then exposed to ultrasonication with a probe for 3 × 15 s at cycle 4 × 10 % and power % set to 65 %. The incubations with bacterial products were performed at 37°C with continuous agitation at 100 rpm for over night incubations and at 170 rpm for various shorter times which are given at the specific experiment. In order to check for activation of PatA by a eukaryotic factor, 25 µl lysates of U937 monocytes or of *Candida albicans* (kindly provided by Donika Kunze, Robert Koch-Institut) were incubated for 1 h at 37 °C with the same volume of sample (or buffer control) and then 25 µl of this mixture was used in the lipolytic assay. Macrophage and yeast cell lysates were generated by the following method. Pelleted cells were resuspended in 7.5 ml 40 mM Tris-HCl (pH 7.5) and vortexed for 10 min with the addition of approximately 10 g of glass beads. After centrifugation at 10000×g for 20 min the supernatant was transferred into a new tube and 1 × protease inhibitor mix was added. Aliquots were stored at -20°C. Depending upon the nature of the experiment, BYE broth, LB broth, or 40 mM Tris-HCl, pH 7.5 (25°C) was incubated, treated like the cultures, and subsequently used as a negative control.

a) Detection of free fatty acids

Free fatty acids as a marker of PLA, LPLA or lipase activities were determined by the means of NEFA-C-kit® (WAKO) according to the instructions of the manufacturer and as described previously (73, 75). Briefly, 10 µl of the reaction mixture was transferred into a fresh microtiter plate and mixed with 50 µl of solution A and after 15 min incubation at room temperature mixed with 100 µl of solution B and again incubated for 15 min at room temperature. The development of a pink-violet colour indicated the presence of FFA (FFA is bound to coenzyme A by the addition of acyl-coenzyme A synthetase and the product is

subsequently oxidized by the addition of acyl-coenzyme A oxidase also leading to the formation of hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase leads to oxidative coupling of an aniline derivative with 4-amino-antipyrine and to the formation of a dye) and was quantified by measuring the absorbance at 550 nm in an absorbance reader in the presence of a fatty acid standard.

b) Detection of GCAT activity

When culture supernatants or cell lysates were assessed for GCAT activity, 50 µl per ml cholesterol in ethanol (10 mg/ml) was added to a DPPG or MPLPG mixture prior to sonication (75). In order to activate PlaC in bacterial culture supernatants and cell lysates, 100 µl reaction mixtures (containing the lipid suspension and the bacterial product) were incubated with 20 µl of culture supernatant from *L. pneumophila plaC* mutants or partly purified zinc metalloprotease. Partly purified protease was obtained by the following method: *L. pneumophila* 130b culture supernatant (OD_{660} =1.8) was concentrated tenfold by ultra filtration (exclusion pore size 30 kD) and subjected to anion exchange chromatography of protease active fractions using a Resource Q column (Amersham Biosciences) followed by gel filtration using a pre packed HiLoad 26/60 Superdex 200 column (Amersham Biosciences). Gel filtration fractions which contained only protease (approximately 2 µg/ml), estimated by SDS PAGE and silver staining were stored at -20°C. A final concentration of 2 mM $ZnCl_2$ was added to the fractions prior use in the activation assays.

c) Detection of 2-acylglycerolphospholipid acyltransferase activity

When the osmotic shock fractions were assessed for 2-acylglycerophospholipid acyltransferase activity, 50 µl/ml palmitic acid in ethanol (3.4 mg/ml) was added to a MPLPG mixture prior sonication and 100 µl/ml T4 DNA ligase buffer (containing 10 mM $MgCl_2$, 10 mM dithiothreitol, 1mM ATP, and 2.5 µg/ml BSA) for supplying the reaction with ATP and $Mg2^+$ was added after sonication.

d) Detection of bacterial membrane lipids

To analyze bacterial membrane lipids, 1 ml of bacterial cultures from late logarithmic phase (OD_{660}=2.0) was lysed as described in chapter 3.2.3 and 100 µl of the cell lysates was used for lipid extraction. For the detection of *L. pneumophila* membrane lipids after growth in the presence of MPLPC, an aliquot of 100 µl from a bacterial liquid culture was pelleted, resuspended in 100 µl of 40 mM Tris-HCl and directly used for lipid extraction.

3.2.4.2 Lipid extraction and thin layer chromatography

For the detection of distinct lipids, reaction mixtures were subjected to a lipid extraction according to Bligh and Dyer (25). For this, 100 µl of reaction mixture were incubated with 400 µl methanol and 200 µl chloroform for 30 min at 37 °C. Then, 280 µl H_2O_{dest} and 200 µl chloroform were added and the mixture was agitated for 10 min at room temperature for a thorough mixing of the phases and subsequently centrifuged for 2 min at 2000×g for phase separation. The chloroform phase was then completely evaporated and the lyophilized product was dissolved in 30 µl of a chloroform/methanol 2:1 (v/v) mixture which was used for separation of lipids by TLC. For detection of cholesterol esters, silica gel plates (Merck, Darmstadt, Germany) were developed in tanks containing an apolar petroleum ether solvent mixture consisting of petroleum ether, diethyl ether, and glacial acetic acid in a ratio of 90:10:1 (v/v/v). For the detection of other non-polar lipids (e.g. glycerolipids, fatty acids) an apolar n-hexane solvent mixture consisting of n-hexane, diethyl ether, and acetic acid in a ratio of 70:30:4 (v/v/v) was employed. For the detection of more polar lipids (e.g. lysophospholipids, phospholipids) a polar solvent mixture of chloroform, methanol, and water in a ratio of 65:25:4 (v/v/v) was used. For visualization, silica plates were briefly put in H_2O_{dest}, then stained for 10 min with 0.2 % (w/v) in 1 M NaCl naphthol blue black and then briefly washed with 1M NaCl (151).

3.2.4.3 Detection of esterase and phosphatase activities

50 µl of culture supernatants or cell lysates were incubated with the same volume of 10 mg/ml p-nitrophenyl butyrate (for the detection of esterase activity) or p-nitrophenyl phosphate (for the detection of phosphatase activity) in 40 mM (pH 7.5) Tris-HCl. In order to inhibit the major acid phosphatase Map, 10 mM sodium tartrate was added along with the p-nitrophenylphosphate (8). The incubations with bacterial products were performed at 37°C with continuous agitation at 150 rpm. Depending upon the nature of the experiment, BYE broth, LB broth, or 40 mM Tris-HCl, pH 7.5 (25°C) was incubated, treated like the cultures, and subsequently used as a negative control. The development of a yellow colour indicated the presence of released p-nitrophenol and was quantified by measuring the absorbance at 405 nm in an absorbance reader in the presence of a standard.

3.2.5 Intracellular infection of U937 cells and *A. castellanii* amoebae

A. castellanii amoebae and U937 (CRL-1593.2, ATCC, Manassas, VA, USA), a human cell line that differentiates into macrophage-like cells upon treatment with phorbol esters (80 nM

phorbol-12-mystrate-13-acetate, P-8139, Sigma Chemicals, Munich, Germany, incubation for 36-48 h), were used as hosts for in vitro infection by *L. pneumophila*. To assess intracellular growth of *L. pneumophila*, wells containing amoebae or U937 cells in a concentration of 10^5/ml and 10^6/ml, respectively, were infected with wild type bacteria or isogenic mutants at a multiplicity of infection of 0.01 (strain 130b) or 0.1 (Philadelpha-1) for amoebal and 1 for U937 cell-infections (0 h time point). Amoebae were incubated for 72 h with the bacteria in infection medium and at intervals of 24 h the amoebae were lysed by pipetting the cells up and down. An aliquot of the infection was then plated on BCYE agar plates. In order to analyze a possible modification of amoebal sterols by *L. pneumophila*, the amoebae from the infection taken at the desired time points were pelleted at 1000 × g and the supernatant was discarded. The pellet was resuspended in 100 µl Tris-HCl (pH 7.5) and lysed by pipetting up and down and subjected to a lipid extraction. Alternatively, the pellet and the supernatant (approximately 1.8 ml) were mixed by pipetting up and down and likewise subjected to a Bligh and Dyer extraction. U937 macrophages were incubated for two hours with the added bacteria in plain RPMI, then monolayers were washed three times with plain RPMI to remove unbound bacteria and were afterwards incubated with RPMI containing 10 % (v/v) fetal calf serum (PAA; Linz, Austria). At 24 h intervals, co-incubations of U937 cells and Legionellae were treated with 10 % (w/v) saponin (Sigma Chemicals, Munich, Germany) for lysis of the host cells and serial dilutions were plated on BCYE agar. For the analysis of U937 lipids, the cells from the desired time points of intection were centrifuged at 800 × g, leaving the majority of extracellular bacteria in the supernatant which was removed. The pelleted macrophages were lysed by saponin treatment to release the majority of the intracellular bacteria into the supernatant and again pelleted by centrifugation at 800 × g. Finally the macrophages were washed with PBS to remove the medium and saponin, resuspended in 100 µl Tris-HCl and used for lipid extraction.

3.2.6 Biochemical protein purification

For the identification of novel PLAs, *L. pneumophila* culture supernatant was partly purified. First 1.2 or 3 l of culture supernatant from mid logarithmic bacteria (OD_{660} = 1.8-2.0) was obtained by centrifugation and was sterile filtered and 5 mM EDTA was added to inhibit metalloprotease activity. Then, the culture supernatant was concentrated tenfold by using the Pellicon XL 50 ultrafiltration device from Millipore (exclusion pore size 30 kDa). The retentate (i.e. proteins >30 kDa) was subjected to anion exchange chromatography (AEX) using the prepacked Resource Q columns from Amersham (1 ml and 6 ml columns for 1.2 l and 3 l of initial culture volumes, respectively). Proteins were eluted by a stepwise increase

of the NaCl concentration: 10%, 20%, 30%, 40%, 50%, and 100% of 1 M NaCl. Fractions of 2 ml or 10 ml were collected and assayed for PLA/LPLA and GCAT activities. Fractions with high PLA activity were then concentrated five-times by using Amicon filter devices (Millipore) with an exclusion pore size of 10 kDa and subjected to gel filtration (GF) by using a prepacked HiLoad 26/60 Superdex 200 column (Amersham Biosciences) (Table 3.1 and 3.6). The amount of protein in each AEX and GF fraction was quantified by means of the Roti Nanoquant solution from Roth and measuring the OD_{595}/OD_{450}. Relevant fractions were also analyzed by SDS PAGE and silver staining. To this purpose, generally 50 ng to 600 ng of protein in a maximum volume of 15 µl was mixed with 1× Roti-Load 1 (reducing protein loading buffer) and boiled for 90s at 95°C and then put on ice until being loaded on a gel. 12.5 % SDS ready gels were used for separating the proteins by electrophoresis at 600 V, 50 mA, and 30 W using the Amersham Multiphor II electrophoresis system (Table 3.1 and 3.2). For N-terminal sequencing, approximately 1µg to 8 µg of protein in a maximum volume of 20 µl was used for SDS PAGE with the Bio-Rad Mini Proten 3 system at 200 V. Protein bands in PLA active AEX and GF fractions were transferred to a PVDS membrane (60 min at 100 V, 350 mA) and visualized by a reversible staining with Ponceau S (Table 3.3). Bands of interest were cut-out and subjected to N-terminal sequencing according to the method of Edman. The N-terminal protein sequencing was performed by the group of Stefan Stevanovic (Universität Tübingen).