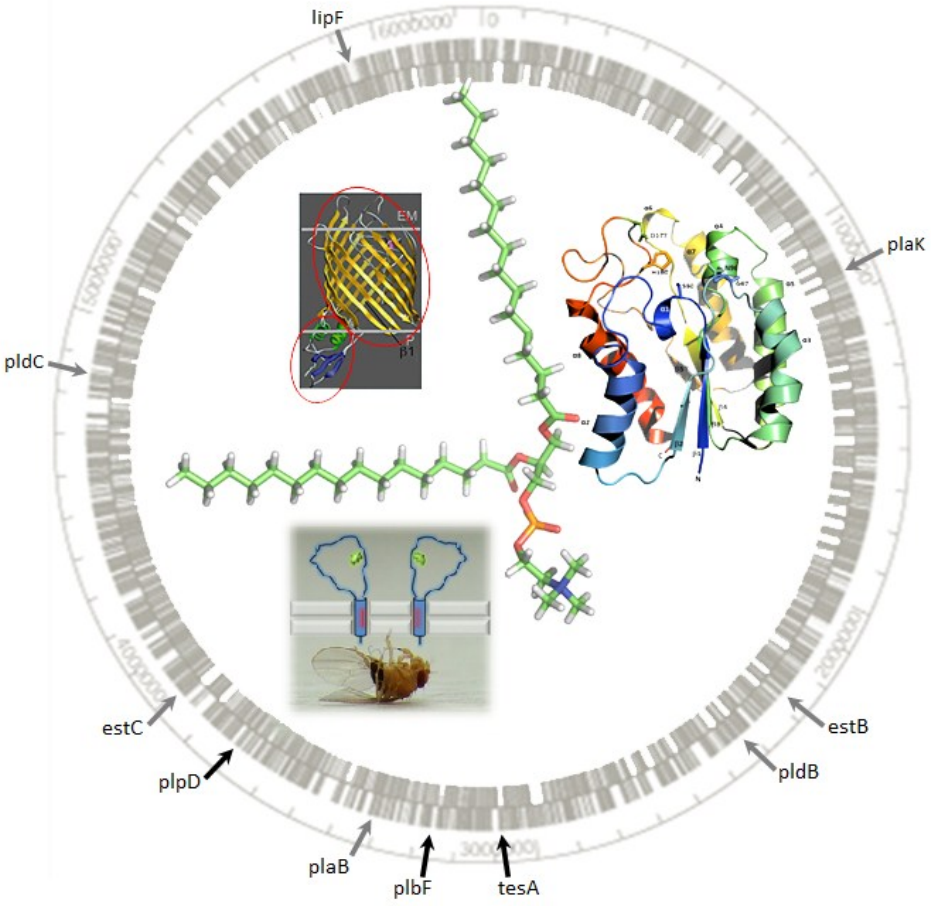
Novel phospholipases A of *Pseudomonas aeruginosa*

biochemical characterisation and cellular localisation



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Thesis for doctoral degree

HEINRICH HEINE
UNIVERSITÄT DÜSSELDORF

Novel phospholipases A of *Pseudomonas aeruginosa*: biochemical characterisation and cellular localisation

Inaugural-Dissertation

zur Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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aus Čakovec in Kroatien

Düsseldorf, June 2010

Aus dem Institut für Molekulare Enzymtechnologie
der Heinrich-Heine Universität Düsseldorf
im Forschungszentrum Jülich

Diese Arbeit wurde gefördert durch ein Stipendium der Europäische Union im Rahmen des Marie-Curie „Early stage research Antibiotarget project“ (MEST-CT-2005-020278).

Gedruckt mit der Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Karl-Erich Jaeger
Koreferent: Prof. Dr. Lutz Schmitt

Tag der mündlichen Prüfung: 23. 06. 201

to Lina and Diana

Acknowledgments

Writing an acknowledgment section is not an easy task as too many amazing people have contributed in various ways in bringing this dissertation about. So let me start by thanking all those people who have helped make my life so amusing and thrilling during the last years.

I would like to thank **Dr. Karl-Erich Jaeger** for giving me the opportunity to join a group and to explore the world of science. Thank you for all the scientific discussions, recommendations and personal understanding.

I want to thank **Dr. Lutz Schmitt** for the kind acceptance of the duty to be the co-referee.

I can't thank my supervisors, **Dr. Susanne Wilhelm** and **Dr. Frank Rosenau**, enough for their bountiful scientific advice and inspiring support. I would also like to thank them for their suggestions which assisted me in preparing the thesis and for the numerous discussions about Pseudomonas, life and how to be a good scientist. Simply, thank you for being such great mentors and good people.

I would like to thank **Dr. Romé Voulhoux** from the Laboratoire d'Ingénierie des Systèmes Macromoléculaires, (IBSM, CNRS, Marseille) for hosting me in his laboratory, providing me with supervision in localization of proteins and for making my stay in Marseille scientifically and socially an outstanding experience. Also I would like to thank **Geneviève Ball** (IBSM, CNRS) for helping in the construction of deletion mutants and for assisting in the laboratory. Thank you **Melissa Sivaneson** and **Astrid Wahl** for enriching my stay in Marseille.

Sincere thanks goes to **Renu Batra-Safferling** and **Joachim Granzin** from the Institute of Structural Biology II (FZ Juelich) for help in the crystallization and the solving of TesA protein structure.

I would like to thank **Dr. Sophie Bleves** and **Richard Salacha** from IBSM (CNRS Marseile) for productive discussions about the secretion and virulence of *Pseudomonas aeruginosa*.

Thanks goes out to **Rien Hoge** from IMET (FZ Juelich) who tested virulence of my *P. aeruginosa* strains in **Dr. Laurence Rahme** laboratory at the Harvard Medical School, Boston.

I would also like to thank **Dr. Melanie Brocker** from the Institute of Biotechnology (FZ Juelich) for help in the identification of proteins by MALDI MS.

My special thanks goes out to **Dr. Silke Isenhardt-Heckmann** (IMET, FZ Juelich) for valuable discussions and commentaries about my Ph. D. thesis and to my colleagues, **Rien Hoge, Sebnem Bukavaz** and **Franco Circolone** (IMET, FZ Juelich) who have been contributing in the development of my scientific thoughts and for helping me in moments of professional and personal happiness. Thank you very much.

I would like to thank all of my fellows and supervisors and all persons involved in the Antibiotarget project for the productive, beautiful and funny moments during the project meetings. It was great to meet you all.

I thank all my friends for making my Ph. D. "adventure" easier.

Last but absolutely not least I would like to thank my family, **mother, father, brothers, wife** and my small angels, **daughters Lina and Diana**, who have been supporting me all the time and giving me the best advice coming deeply from their hearts.

Publications in frame of PhD thesis

Articles

Accepted: R. Salacha, **F. Kovačić**, B.-A. Celine, S. Wilhelm, J. Tommassen, A. Filloux, R. Voulhoux, and S. Bleves (2010) *The Pseudomonas patatin-like protein PlpD is the archetype of a novel Type V Secretion System*, Environmental Microbiology, Environmental Microbiology. **12**(6): 1498-1512.

: I. Leščić Ašler, N. Ivić, **F. Kovačić**, S. Schell, J. Knorr, U. Krauss, S. Wilhelm, B. Kojić-Prodić and K.-E. Jaeger (2010) *Probing enzyme promiscuity of SGNH – hydrolases*, ChemBioChem. **11**(15): 2158-67.

In preparation: **F. Kovačić**, F. Rosenau, S. Wilhelm, K.-E. Jaeger, *Diversity of novel phospholipases A from* Pseudomonas aeruginosa.

: **F. Kovačić**, R. Batra-Safferling, F. Rosenau, J. Granzin, B. Kojić-Prodić, K.-E. Jaeger, S. Wilhelm, *Structural and functional characterisation of TesA, a periplasmic lysophospholipase from opportunistic pathogen* Pseudomonas aeruginosa

Poster presentations

April 2007, Conference of German Association for General and Applied Microbiology, Osnabrueck, Germany, **F. Kovačić**, S. Wilhelm, F. Rosenau and K.-E. Jaeger, Characterisation of the lipolytic system of Pseudomonas aeruginosa.

June 2007, Tag des wissenschaftlischen nachwuches, Düsseldorf, Germany, **F. Kovačić**, S. Wilhelm, F. Rosenau and K.-E. Jaeger, *Characterisation of lipolytic system of the pathogenic bacterium* P. aeruginosa.

August 2009, XII International Pseudomonas Conference, Hannover, **F. Kovačić,** A. Mandrysch, S. Wilhelm, F. Rosenau, K.-E. Jaeger, Pseudomonas aeruginosa *PAO1 expresses enzymes with phospholipase A and phospholipase B activity*.

August 2009, XII International Pseudomonas Conference, Hannover, R. Salacha, **F. Kovačić**, J. Tommassen, A. Filloux, R. Voulhoux, and S. Bleves, *An original mechanism for the secretion of a patatin-like protein by* Pseudomonas aeruginosa.

Oral presentations

November 2006, First Antibiotarget symposium, Nottingham, England, **F. Kovačić**, S. Wilhelm, F. Rosenau and K.-E. Jaeger, *Characterisation of the lipolytic system of* Pseudomonas aeruginosa.

October 2007, Second Antibiotarget symposium, Jülich, **F. Kovačić**, S. Wilhelm, F. Rosenau and K.-E. Jaeger, *Characterisation of the lipolytic system of* Pseudomonas aeruginosa.

May 2008, Third Antibiotarget symposium, Marseilles, France, **F. Kovačić**, S. Wilhelm, F. Rosenau and K.-E. Jaeger, *Characterisation of the lipolytic system of* Pseudomonas aeruginosa.

December 2009, Fifth Antibiotarget symposium, London, England, **F. Kovačić**, S. Wilhelm, F. Rosenau and K.-E. Jaeger, *Characterisation of the lipolytic system of* Pseudomonas aeruginosa.

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List of Abbreviations

In this PhD work were used abbreviation accepted from International System (SI) of Units

Mw	Molecular weight
Da	Dalton
PPGAS	Phosphate limited Protease peptone Glucose Ammonium salt
LB	Luria Bertani
IPTG	Isoprppyl-ß-D-thiogalactopyranoside
X-Gal	5-Bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside
DMF	Dimethylformamide
EDTA	ethylenediaminetetraacetic acid
MES	2-(N-morpholino)ethanesulphonic acid
Amp	Ampicillin
Cm	Chloramphenicol
Tc	Tetracyclin
Sm	Streptemycin
Irg	Irgasan
Cb	Carbenicillin
Spc	Spectinomycin
Gm	Gentamycin
DMSO	Dimethyl sulfoxide
MUB	4-Methylumbelliferyl Butyrate
aa	Amino acid
P_{lac}	Lactose operated promoter
MME	Minimal medium E
M9	Minimal 9
3D	Three dimensional
U_{Cell}	Unit of cells, equivalent of OD_{580nm} (chapter 3.1.1)
HRP	Horseradish peroxidise
AA	Arachidonic acid
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
w/v	weight per volume
v/v	volume per volume
AI	Autoinduction
A. dest.	*Aqua destillatum*
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
CoA	Coenzyme A
pNPP	*p*-nitrophenyl palmitate
pNPC	*p*-nitrophenyl caproate
TNB	5-thio-2-nitrobenzoat
NMR	Nuclear magnetic resonance
MALDI MS	Matrix assisted laser dissociation-ionization mass spectrometry
PAF	Platelet activating factor
SFGH	S-formylglutation hydrolases
HSL	Hormone sensitive lipase
2-AG	2-arachidonoylglycerol
PMSF	Phenylmethylsulphonyl fluoride
THL	Tetrahydrolipstatin
TX-100	Triton X-100
NLS	N-lauroyl sarcosine

PLA	Phospholipase A
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
LysoPLA	Lysophospholipase A
LysoPLC	Lysophospholipase C
LysoPLD	Lysophospholipase D

1. Introduction

1.1. *Pseudomonas aeruginosa*, a medical relevant bacterium

The genus *Pseudomonas* represents a diverse group of medical, environmental and biotechnological important bacterium [6]. One member of this genus, namely *P. aeruginosa*, differs from others because it is versatile opportunistic pathogen causing infections in mammalians, plants, yeasts and insects [7]. Thus, *P. aeruginosa* is the most common *gram-negative* bacteria found in nosocomial infections [8]. It has the ability to infect various subpopulations of immunocompromised patients, particularly burn victims, cystic fibrosis, AIDS and cancer patients [9]. *P. aeruginosa* causes death with a rate of 30-60% in pneumonia and septicaemia [10]. The sequencing of the *P. aeruginosa* PA01 [11] genome confirmed its large size suggested before the genome was known. Thus, with ca. 5600 predicted open reading frames the *P. aeruginosa* genome closely approaches to the size of the simple eukaryote *S. cervevisiae* (6200 genes) [11]. The *P. aeruginosa* genome is rich in genes encoding transporters and regulatory proteins [11] what is in consistence with environmental adaptation and particular virulence potency of the strain.

The pathogenicity of *P. aeruginosa* lies in the incredible ability to produce many cell associated (lipopolysacharides, flagellum, type IV pili, alginate) and secreted (proteaseses, exotoxines, rhamnolipids, pyocyanin, phospholipases) virulence factors [8]. Both, *in vivo* production and secretion of virulence factors is to some extent, as much as we understand today, regulated by cell-to-cell signalling system popularly called “quorum sensing” [8, 12, 13]. In general bacteria sense microenvironment and once in a host they response by production of virulence factors [14]. Changing of virulence factor arsenal during the shift from colonisation phase to acute or chronic infection point out *In vivo* regulation of virulence factors production by *P. aeruginosa* [8]. Today, numerous reports about genetic adaptation of *P. aeruginosa* during long-term infections are existing. These adaptations involve mutations in genes encoding transporter, regulatory and virulence proteins [15]. However, it is not unusual that only certain bacteria in one population carry a particular mutation that is not present in other bacteria [16, 17]. This, together with importance of secretion for virulence of *P. aeruginosa*, is strengthened by the identification of striking differences in extracellular proteome of different *P. aeruginosa* isolates of the same cystic fibrosis patient [18]. Taken together, it seems that cooperation of members of bacterial population, which may have a distinct function, may be beneficial for the whole population and finally may have consequences for the development of *P. aeruginosa* infection.

Thus, the pathogenicity of *P. aeruginosa* seems to be a complex issue because of dynamic production and secretion of virulence factors and genetic diversity of bacteria in a population. Beside above underlined complexity of *P. aeruginosa* pathogenicity, we would like to stress the fact that 43% of *P. aeruginosa* PA01 genes encode for the hypothetical proteins and proteins with putative function [19]. As a consequence we are fully limited in understanding of anyway complex behaviour of *P. aeruginosa* during the infections. Thus, novel antibiotics targets and novel approaches in treatment of *P. aeruginosa* infections are today on the frontier of science [14].

1.2. Lipolytic enzymes of *P. aeruginosa* involved in pathogenesis

A number of lipolytic enzymes including phospholipases C (PlcH, PlcN, PlcB), phospholipase D (PldA), phospholipase A2 (ExoU), lipases (LipA, LipC) and esterase (EstA) are known that may contribute to the virulence of *P. aeruginosa* (Table 1). However, mechanisms and roles during the infection and pathogenesis for most of them are still not elucidated. From this chapter are omitted ExoU and EstA which will be presented later.

Table 1: Known lipolytic enzymes of *P. aeruginosa* PAO1.

PA code	Gene name	Product name	Reference
PA0026	*plcB*	phospholipase C, PlcB	[20]
PA0843	*plcR*	phospholipase accessory protein PlcR precursor	[21]
PA0844	*plcH*	hemolytic phospholipase C precursor	[22]
PA2862	*lipA*	lactonizing lipase precursor	[23]
PA2863	*lipH*	lipase modulator protein	[24]
PA3319	*plcN*	phospholipase C precursor	[25]
PA3487	*pldA*	phospholipase D	[26]
PA4813	*lipC*	lipase LipC	[27]
PA5112	*estA*	esterase EstA	[28]
PA3859	-	Carboxylesterase	[29]

P. aeruginosa produces and secretes three phospholipases C; PlcH, PlcN, PlcB. Only PlcH possesses haemolytic activity what classifies it like a potential virulence factor. And indeed it was demonstrated that PlcH is produced during clinical infection [30] and that purified PlcH injected into mice cause tissue necrosis [31, 32]. Reduced virulence of *P. aeruginosa* strain negative in *plcH* gene in mouse burn models refers involvement of PlcH in *P. aeruginosa* pathogenesis *In vivo* [33]. In rabbit lung infection model were observed lower levels of inflammatory mediators after the infection with *plcH* deletion mutant compared to the wild type [34]. Furthermore, mice virulence model reveals that purified PlcH *In vivo* stimulates production of inflammatory mediators by peritoneal macrophages [35]. The experiments with *plcH* deletion mutant and with purified PlcH imply the capability of PlcH to interfere with lipid signalling processes in eukaryotic cells. The finding that transcription of nonhemolytic PlcN gene is up regulated during the interaction of human airway epithelia cells with *P. aeruginosa* indicates its possible role in lung infection [36]. Even PlcB [20] exhibits a broad range phospholipase activity its involvement in *P. aeruginosa* pathogenesis is still not confirmed. The interesting phenotype associated with PlcB is the observation that it is essential for chemotaxis toward phospholipids what could be important in dissemination of bacteria in the infected tissue in direction of nutrient rich habitat [37].

Although, *P. aeruginosa* infection is related with lung tissue destruction [38] and it was suggested that lung surfactant is degraded *In vivo* during the infection [39] involvement of lipases in *P. aeruginosa* pathogenesis is still unclear. However, it seems that extracellular lipase (LipA) [23] enhance a phospholipase C induced release of some inflammatory mediators from the inflammatory cells [40]. Since LipA by itself could not liberate the same mediators, but phospholipase C could, it was suggested that simultaneous secretion of these virulence factors might be of benefit for *P. aeruginosa* infection [40]. Indeed, later in the microarray study of *P. aeruginosa* isolated from cystic fibrosis patients both, *plcH* and *lipA*, were suggested to contribute to the lung surfactants lipid

degradation [39]. For LipC, the second secreted lipase of *P. aeruginosa*, was proposed a role in utilisation of exogenous lipids or the modulation of inflammatory lipid mediators during the infection but evidence supporting this suggestion does not exist [27, 41].

Phospholipases D (PLDs) are found in only a very limited number of prokaryotic organisms in which they play a role in bacterial pathogenesis. In *P. aeruginosa* only one phospholipase D (PldA) is detected. It is a periplasmic protein involved in persistence of bacteria during the infection but it is not known whether it acts as a toxin on the host cells or if it affects physiology of *P. aeruginosa* required for the long-term survival [26].

1.3. Phospholipases

1.3.1. Definition and classification of phospholipases

Biochemical definition

Phospholipases constitute a heterogeneous group of enzymes that share the ability to hydrolyze one or more ester bond in glycerophospholipids [42].

Physiological definition

Phospholipases are ubiquitous and diverse enzymes that mediate various cellular functions including membrane maintaining, cellular turnover and the generation of inflammatory response [43].

It is notable that both definitions are somewhat uncertain dealing with terms like: “heterogeneous group”, “diverse enzymes” and “one or more bonds”. This inability to precisely define a phospholipases (PLs) originats from the nature of their substrate, a glycerophospholipid, which possesses four ester bonds and may be degraded by phospholipases to many distinct classes of molecules that comprise biologically active compounds. The four different phospholipid cleavage sites, in other words, four ester bonds of phospholipid allow the classification of phospholipases (PL) into four major groups, phospholipases A, B, C and D (Figure 1) [43]. Furthermore, PLA may be divided in two sub-groups, PLA1 (EC 3.1.1.32) and PLA2 (EC 3.1.1.4), which catalyze the hydrolysis of phospholipids at the sn1 and sn2 position respectively yielding a free fatty acid and the lysophospholipid. Lysophospholipids are substrates of lysophospholipases (LysoPLA) which convert it to the glycerophosphocholine and fatty acid (EC 3.1.1.5). Two different lysophospholipids may be produced by the activity of PLA1 (2-acyl lysophospholipid) and PLA2 (1-acyl lysophospholipid). Therefore, lysophospholipases are also classified in two subgroups, LysoPLA1 and LysoPLA2 according their preference for sn1 or sn2 position [44]. The phospholipases with the same preference for sn1 and sn2 position are called PLB, their function overlap with the function of LysoPLA, PLA1 and PLA2 and therefore it is not an accepted name by EC but still it is common name in the literature. The enzymes hydrolysing the phosphoester bounds of phospholipids are called PLC (EC 3.1.4.3) and PLD (EC 3.1.4.4). Analogously to PLC and PLD, the enzymes that hydrolyse phosphoester bounds of lysophospholipids are called LyosPLC [45] and LysoPLD [46]. However, it might be mentioned that LysoPLC and LysoPLD are rare enzymes detected only in eukaryotes.

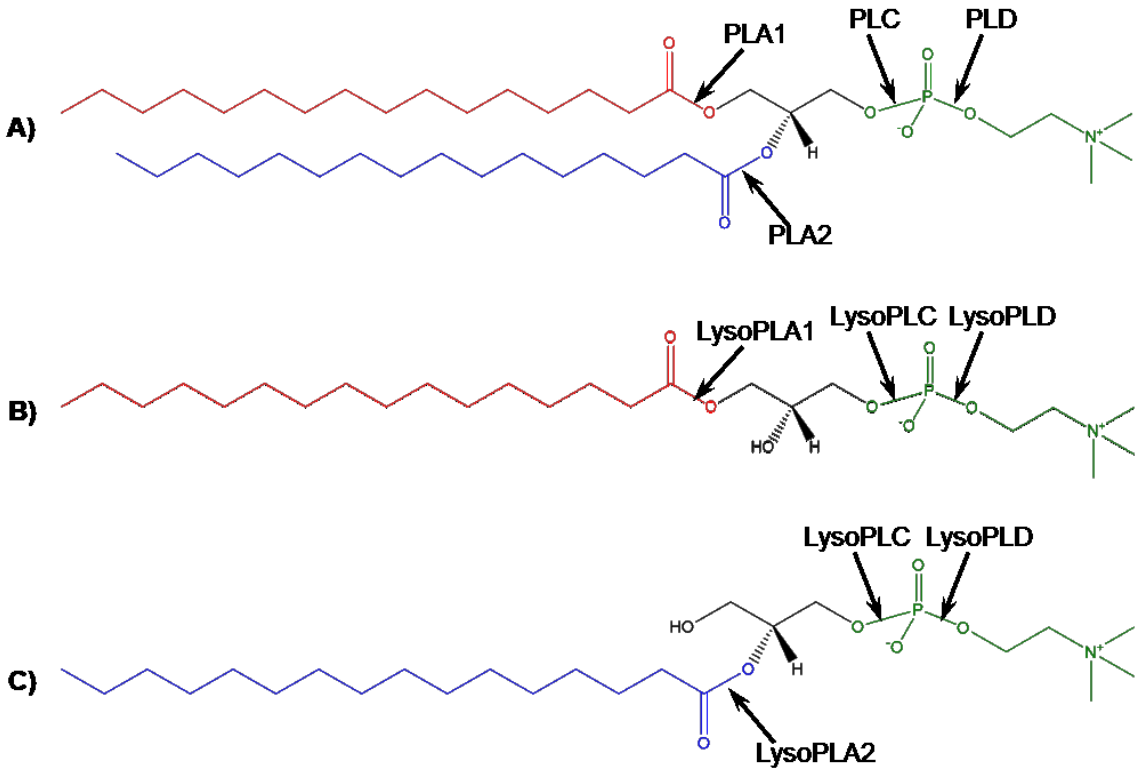


Figure 1: Classification of phospholipases based on the cleavage of different ester bonds of phospholipid (A) and lysophospholipid (B, C) molecules. PLA1, phospholipase A1; PLA2, phospholipase A2; PLB, phospholipase B; PLC, phospholipase C; PLD, phospholipase D; LysoPLA1, lysophospholipase A1; LysoPLA2, lysophospholipase A2; LysoPLC, lysophospholipase C; LysoPLD, lysophospholipase D.

Additional confusion in phospholipase terminology cause esterase (EC 3.1.1.1) or lipase (EC 3.1.1.3) activity demonstrated for number of phospholipases A and B or vice versa [42]. Esterases and lipases hydrolyse, respectively, water-soluble and water-insoluble neutral lipids (without charge) like mono-, di- and triacylglycerols [47, 48]. Thus, some enzymes discovered like lipases and esterases and later proven as a phospholipases are rather classified as a lipase/esterase than phospholipase.

Furthermore, for some phospholipases were reported additional phosphodiesterase (EC 3.1.4.1) [42], acyltransferase (EC 2.3.1.26) [49] or acyl-CoA thioesterase (EC 3.1.2.1 and EC 3.1.2.2) [50] activity. These factors probably are responsible for lack of systematic classification of phospholipases even some partial classification of mammalian phospholipases are existing [51, 52].

A range of natural substrates hydrolysed by phospholipases broaden the space of cellular processes in that they can contribute. Such broad substrate ranges of phospholipases make them attractive for biotechnological application.

1.3.2. Three-dimensional structures of prokaryotic phospholipases A

The biological function of proteins is related to their molecular structures therefore it will be introduced some structural details of bacterial PLA. A limited number of bacterial PLAs are known therefore up to date are available only three crystal structures of enzyme from this class (Table 2). Although, *E. coli* lysophospholipase [3] does not exert PLA activity it is mentioned in table 2 as an enzyme involved in phospholipid hydrolysis. Two PLAs originate from Gram-positive bacteria, *S. violaceoruber* and *S. hyicus*, and one from *Gram-negative* bacterium (*E. coli*). The structure of outer membrane PLA (OMPLA) will be used to explain some possible physiological effects of OMPLA in the next chapter.

Table 2: Bacterial (lyso)phospholipases A with known crystal structure.

PDB ID	Enzyme function	Biological source	EC number
1LWB	Phospholipase A2	*Streptomyces violaceoruber*	3.1.1.4
2 hIH	Phospholipase A1	*Staphylococcus hyicus*	3.1.1.3
1QD6	Outer membrane PLA	*Escherichia coli*	3.1.1.32
1IVN	Lysophospholipase	*Escherichia coli*	3.1.1.5

Secreted PLA2 from *S. violaceoruber* PLA2 (SvPLA2) is the first structurally characterised prokaryotic PLA2 [54]. PLA2 are commonly found in eukaryotes where they carry plethora of important cellular functions thus numerous eukaryotic PLA2 are identified to date [55, 56]. However, striking differences of SvPLA2 to eukaryotic PLA2s were observed in primary sequence, calcium binding constant, number of disulfide bonds and overall protein fold [57]. Therefore, SvPLA2 was proposed as a member of novel group of PLA2, bacterial PLA2 [57]. All secreted eukaryotic PLA2 require calcium for proper functionality. The activity of SvPLA2 is also calcium dependent but the calcium binding constant of SvPLA2 is one order of magnitude lower compared to the eukaryotic PLA2s [57]. Eukaryotic PLA2s are characterised by 6-8 disulfide bounds in the structure when in the SvPLA2 structure are recognised only 2 disulfide bounds [54]. Furthermore, in the structures of eukaryotic PLA2s were recognised α-helices and β-strands what is opposite to the all α-helix structure of SvPLA2 [54].

Despite underlined dissimilarities of SvPLA2 and eukaryotic PLA2s, the structural comparison of SvPLA2 and bovine pancreatic PLA2 [58] revealed their partial structural conservation [59]. The His64 and Asp85 form an active site of SvPLA2 together with water molecule which serves as a nucleophile in hydrolysis of phospholipids substrates [59]. The member of active site His64 and additionally Asp65, Asp43 and Leu44 are bound to the calcium ion in vicinity of active site [57]. Exactly, calcium binding residues, catalytic residues and residues in direct contact to them are structurally invariant in SvPLA2 and bovine PLA2 (Figure 2) [59].

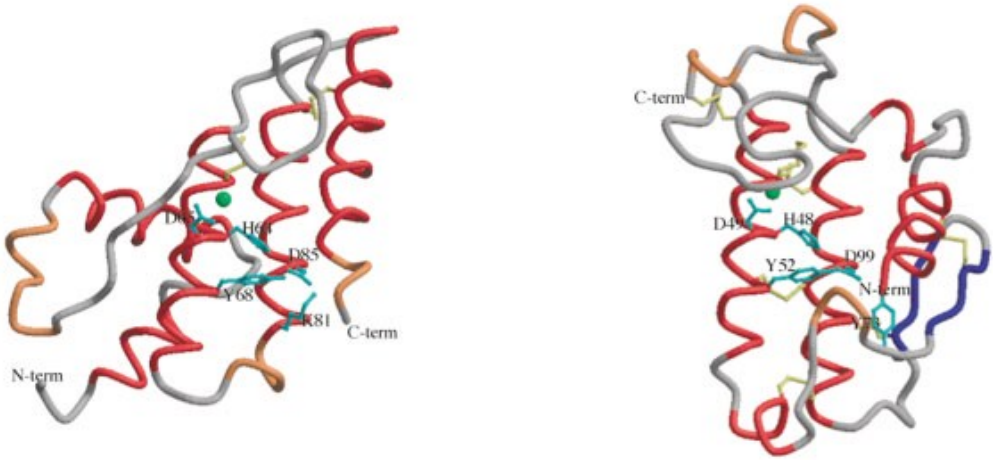


Figure 2: The overall structures of *S. violaceoruber* PLA2 (left) and bovine pancreatic PLA2 (right). Secondary structures, α-helices, 3_{10}-helices and β-strands are coloured in red, orange, and blue respectively. Disulfide bonds are indicated by yellow colour. Calcium ions are represented as a green spheres. Structural invariant residues forming the catalytic dyad and ligating to the calcium ion and the residues directly interacting with the catalytic site are depicted in green. Figure is adopted from Matoba *et al.* (2003) [59].

The interfacial activation is common feature of eukaryotic PLA2s, and it was demonstrated for SvPLA2 [59, 60]. Thus, it was suggested stabilization of active sites of eukaryotic PLA2s upon interaction with insoluble substrates. This was confirmed by observed particular flexibility of active site and phospholipid interaction regions in solution (by NMR) but not in the solid state [58]. Interestingly, SvPLA2 structures solved at different temperatures confirmed flexibility of the same regions, active site and its neighbourhoods, as was observed for bovine PLA2 [59, 54]. Thus, the role of hydrogen-bonds network in stabilisation of active sites of eukaryotic PLA2s and SvPLA2 was proposed as a general mechanism of the interfacial activation [59]

The physiological function of SvPLA2 was proposed based on the enzyme specificity for phosphatidylcholine over the phosphatidylethanolamine [57]. *Streptomyces* membranes are not composed of phosphatidylcholine opposite to some other bacteria [57, 61]. Therefore, it might be that soil bacterium *S. violaceoruber* produces secreted PLA2 as a toxin against bacteria containing phosphatidylcholine in the membranes. It could represent a surviving mechanism in the ecological niche occupied by different microorganisms as it is soil [57]. Indeed, PLA producer organisms have developed different mechanisms to protect themselves from the action of their own PLAs. Thus, human PLA2 found enriched in rheumatoid synovial fluid exerts striking preference for *E. coli* membrane lipids [62]. Another example is *P. aeruginosa* which secretes inactive PLA2 (ExoU) directly in the host cells where it become active in the contact with eukaryotic effector [63].

S. hyicus PLA1 (ShPLA1) is an example of enzyme discovered long time ago as a lipase [64] and later reported as a PLA1 [65]. Therefore, ShPLA1 is still referred as a lipase in literature. However, it differs from other bacterial lipases in its high PLA1 activity beside its considerable lipase activity [66]. The role of Lys295 and Ser356 for determination of PLA1 activity of ShPLA1 was suggested using the site directed mutagenesis methods [67]. Later, three dimensional structure of ShPLA1 revealed a role of Lys295 in binding of phosphate group of phospholipids, and role of Ser356 in positionating a side

chain of Lys295 in proper position for interaction with phospholipid (Figure 3) [66]. Furthermore, structural analysis of ShPLA1 homologues *G. stearothermophilus* lipases L1 [68] and P1 [69], that show lipase but lack PLA1 activity, revealed conservation of Lys295 and Ser356 of ShPLA1 with the alanine and leucine respectively. This finding suggested chemico-physical properties which distinguish active sites of lipases and phospholipases.

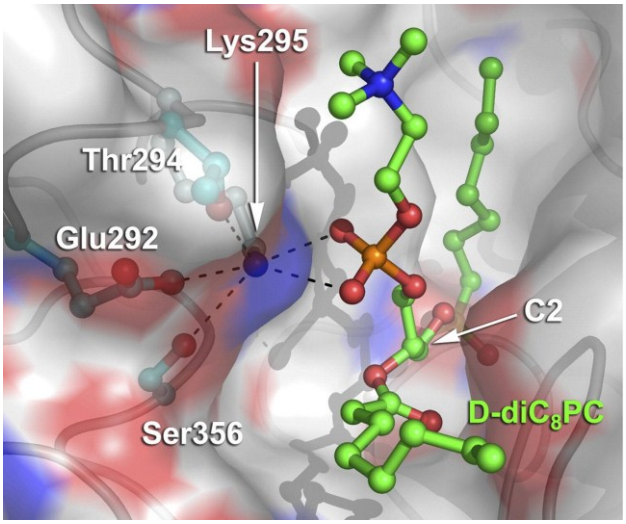


Figure 3: Active site of *S. hyicus* lipase with modelled dioctanoyl phosphatidylcholine (D-diC_8PC) in it. The determinants of SHL phospholipase activity, Lys295, Glu292, Thr294 and Ser356 are depicted with carbon atoms in cyan. Electrostatic interactions between the side-chain of Lys295 and surrounding residues, as well as interactions with the substrate phosphate oxygen atoms are shown as black dashes. Phosphate atom is colored in orange. Red and blue transparent surface represent oxygens and nitrogens respectively. Figure is adopted from Tiesinga *et al.* (2007) [66].

This structural study proves a general opinion that the active sites of phospholipases A are more polar then the active sites of lipases since phospholipids are more polar than lipids. However, in literature are existing further examples of lipases with PLA activities [70] but without clear structural evidences for such specificity. Therefore, it is growing emergency of fine structural details which should support in particularly one of those mechanisms (PLA1, PLA2, lipase and LysoPLA) for better understanding a lipolytic family of lipases and phospholipases.

1.3.3. Physiological functions of bacterial phospholipases A

Phospholipases A are involved in housekeeping cellular processes as membrane remodelling [71], lipid metabolism [72] and nutrients utilisation [39]. One of the best-characterised PLA family is an outer membrane PLA (OMPLA) family detected in numerous pathogen and non-pathogen bacteria [73]. First identified OMPLA is the one from *E. coli* [74] latter shown to be an integral membrane protein [75]. It is constitutively expressed in an inactive form since no phospholipid turnover was detected under physiological conditions [76]. However, *In vivo* perturbation of bacterial membranes, by heat shock, EDTA or colicin release, trigger the activation of OMPLA [77]. *In vitro* activity of OMPLA depends on type and concentration of detergent and on the concentration of OMPLA [78]. This activation occurs through its dimerisation [79]. Dimerisation is reversible process *In vivo* suggested as a part of regulatory mechanism [80]. The reported inactive OMPLA dimer in lipid vesicles point out the proper assembly of two OMPLA molecules necessary for its activation [81].

The crystal structure of OMPLA [80] in a monomeric form revealed 12 stranded antiparallel β-barrel structure that is embedded in the membrane (Figure 4). Active site presented by His142, Ser144 and Asn156 is located at the surface of the extracellular side of barrel. Furthermore, the calcium was demonstrated to be essential for activity of OMPLA since it is required for dimerisation [82]. Crystal

structure of OMPLA dimer revealed that each of two calcium ions interacts with both OMPLA molecules thus inducing a dimerisation [80]. Interestingly, calcium ions were found in vicinity of active sites where they may contribute to the binding and positioning of the substrate in the active site or even to stabilise catalytic intermediary [80]. Indeed, it was demonstrated that substrate binding is related to the dimerisation of OMPLA [76]. Thus, shared calcium binding site explains why OMPLA is active only as a dimer.

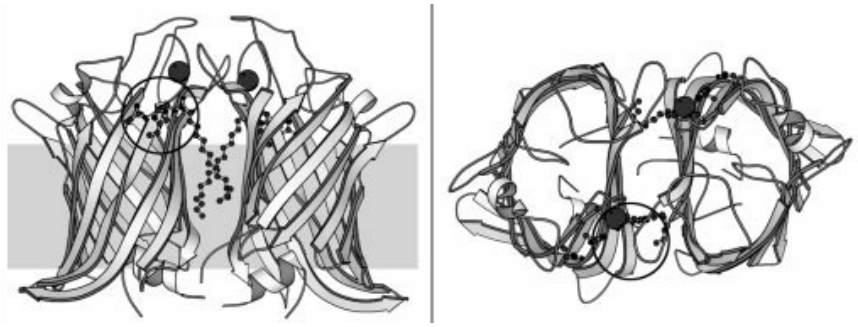


Figure 4: Crystal structure of OMPLA from *E. coli* [80]. Side view (left) and top view (right) of the dimeric complex of OMPLA with the inhibitor, shown in ball-and-stick representation, located between two β-barrels. The active site residues, Asn156, His142 and Ser144 represented as ball-and-stick, are indicated by the circle. Calcium ions bound at the interface are shown as gray spheres. Top of protein is cell-surface exposed, and the bottom is periplasmic exposed. Membrane is represented by the gray area. Figure is adopted from Dekker (2000) [82].

Although OMPLA is comprehensively molecular characterised its function in the cell is less clear. It is suggested that OMPLA has a housekeeping function based on its tightly controlled activity by substrate inhibition what is typical for housekeeping enzymes [76]. Furthermore, OMPLA is responsible for raising of the lysophospholipid content from 2% to over 50% of total membrane lipids in pathogenic *H. pylori* [83]. Thereby, OMPLA increases pH tolerance and attachment of bacteria to the host cells [83]. Therefore some OMPLAs of pathogen bacteria are assumed as virulence factors what is in keeping with observation that OMPLA mutants of some pathogen bacteria are less virulent [42]. However, the enhancement of bacterial growth and colonisation are thought to be related to OMPLA virulence rather than direct effect on the host cell membranes [43]. Generally, it is difficult to distinguish between direct role of PLA2 in virulence of pathogen bacteria and their roles in metabolic processes indirectly influencing bacterial virulence. These may be underlined by the fact that some PLAs display cytotoxic and proinflammatory effect to the host cells but in parallel increase colonisation and fitness of pathogen bacteria [43]. Therefore, it is rational to think about the conserved role of PLAs among pathogen and non-pathogen bacteria related to the bacterial fitness, but additional role of PLAs in pathogen bacteria as a virulence factors.

1.3.4. Role of bacterial phospholipases A in pathogenesis

The possibility that phospholipases A could be generally involved in bacterial pathogenicity was just recently suggested [42]. Doubtless evidences about the involvement of PLA in bacterial virulence and pathogenesis are existing, but lack of purified and characterized enzymes from pathogen or nonpathogenic bacteria obstruct determination of their precise functions in pathophysiological processes.

Haemolysis is the most common and well-understood mechanism of action of phospholipases A during the infection. Examples of haemolytic PLAs are existing in *Campylobacter coli, Vibrio parahaemolyticus, Legionella pneumophila, Rickettsia prowacekii and rickettsii* [84]. Membrane bound lysophospholipids generated by activity of PLA induce membrane expansion and thus contribute to the physical disintegration of the erythrocytes membranes [85].

Furthermore, lysophospholipids at low concentration induce an osmotic lysis of erythrocytes by liberating cell surface polypeptides from erythrocyte membranes what increases permeability for sodium ions [86]. The lysophospholipids can cause lysis of other cells than erythrocytes too as it was observed for cells of gastric epithelium under the action of lysophospholipids produced by *Helicobacter pylori* [87, 88]. The generated lysophospholipids do not affect only cell membrane lipid bilayer integrity but also the integrity of lipid/protein monolayer like stomach mucus [87, 88] and lung surfactant [89]. Thus PLA of respiratory pathogen *L. pneumophila* destroys lung surfactant which is important lipid/protein layer in alveoli with the function to reduce the breathing effort [89]. It was suggested involvement of PLA of *Helicobacter pylori* in ulcer formation trough its degradation of stomach mucus lipids what make mucus less hydrophobic and therefore more susceptible to lysophospholipids [87, 88]. Recently, the synergistic effect of lysophospholipids and dirhamnolipid of *P. aeruginosa* on membrane permeabilisation was reported [90]. Additionally, *P. aeruginosa* can react on the presence of phagocytic cells polymorphonuclear neutrophilic leukocytes (PMNs) increasing production of rhamnolipids [91] which are responsible for rapid killing of PMNs [92]. However, there are no *In vivo* evidences of jointly action of rhamnolipids and lysophospholipids produced by phospholipases A in killing of PMNs.

Lipids are recognised as important eukaryotic signalling molecules. In literature, it is described that mammalian PLA2 releasing arachidonic acid and lysophospholipides from eukaryotic membranes to provide the metabolites for various inflammatory and other important pathways in eukaryotes [93]. Thus, lysophospholipids activate the G protein-coupled receptors present on the surface of T and B cells, macrophages and monocytes which all contribute to the host immune response. Furthermore, lysophospholipids are the precursors in biosynthesis of a platelet activating factor (PAF) which has been implicated in pathophysiological conditions like allergic asthma, endotoxin shock, acute pancreatitis and dermal inflammations [94]. The phosphatidylserine (PS) and lysoPS exposed at the surface of dying cells are recognized by the receptors displayed by the phagocytes what is the signal for phagocytosis [95].

The most common fatty acid linked to sn2 position of eukaryotic phospholipids is arachidonic acid (AA) at the same time it is the most physiologically relevant fatty acid [43]. AA is precursor to a large family of pro-inflammatory eicosanoids which include prostanoids, lipoxines and leukotrienes (Figure 5) [96]. The cellular responses initiated by release of AA by activity of PLA2 result in synthesis of 13

different lipid mediators [97]. Since it was observed that eicosanoids biosynthetic pathways are incomplete in some cell types, the idea about transcellular synthesis of eicosanoids arise. It implies the release of AA from one type of cells (donor cell) and transports of eicosanoids intermediaries to the second cell to complete the biosynthesis of final active compound [97]. Furthermore, the cellular responses mediated by these molecules are associated with physiological as well with pathological conditions (cystic fibrosis, bronchial asthma, atherosclerosis, thrombosis) [98].

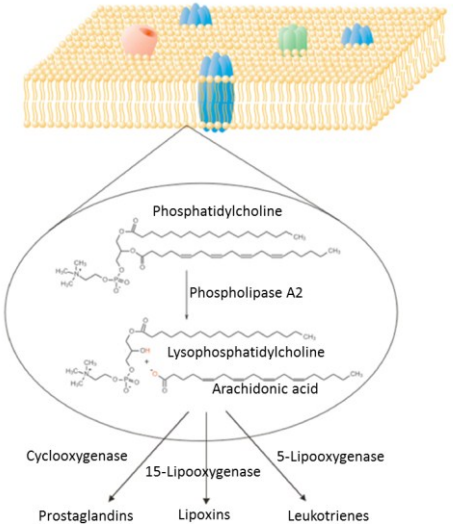


Figure 5: Schematic representation of phospholipase A2 triggered inflammatory response in eukaryotes. PLA2 cleave phosphatidylcholine of eukaryotic membranes releasing arachidonic acid which is precursor in synthesis of inflammatory mediators; prostaglandins, lipoxins and leukotrienes.

Just little is known about direct action of bacterial PLA2 on the host. However, there are some evidences that bacterial PLA2 are involved in the modulation of host inflammatory response and cell signalling pathways. It was demonstrated that PLA2s, ExoU of *P. aeruginosa*, YplA of *Yersinia enterocolitica* [87] and SlaA of *Streptoccocus sp.* [99] are involved in the modulation of host lipid signalling what moreover confirm assumption that the release of bioactive lipids might be of significance for bacterial pathogens.

The gene *yplA* of *Yersinia enterocolitica* encodes a phospholipase A2 [100] which is secreted by type III secretion system [101, 102]. *Yersinia* sp. infect the organism trough gastrointestinal tract where penetrate the epithelium cells trough Pyers patches located in the wall of the intestine. The observation that *yplA* deletion mutant was not recovered in high number as wild type from Peyers patches point out the requirement of YplA for efficient cellular invasion and colonization of pathogen. Furthermore, the necrosis of Peyers patches was increased in organism infected with wild type comparing to the *yplA* deletion mutant indicating stimulation of the acute inflammatory response by YplA [87].

In a group A Streptococcus (GAS) was observed that M3 serotype is associated with unusually severe infections and high mortality rate [103]. Sequencing of the genome of M3 GAS strains reveals existence of novel toxin SlaA [104] with homology to PLA2. Indeed SlaA is secreted PLA2 which expression is greatly induced upon the contact with epithelia cells and human saliva [104]. The infected humans produce antibodies against SlaA what indicates that enzyme is expressed *In vivo* during the course of human disease [103]. SlaA *in vitro* hydrolyses membrane phospholipids releasing the arachidonic acid from sn2 position [99] what may be relevant for modulation of host inflammatory response *In vivo* [43]. Recently the experiments with *slaA* deletion mutant strain

confirmed that SlaA is indeed a virulence factor. Using the human trancheobronchial epithelia cells infection model was demonstrated that *slaA* mutant kills fewer cells than the wild type. Furthermore, the results in mice infection model show that *slaA* mutant is less virulent than wild type. Additionally, decreases of host-cell viability after expression of SlaA in yeast and localization of SlaA inside the epithelial cells after infection with wild type M3 GAS pointed out the intracellular localization of SlaA necessary for its cytotoxic effect [105].

ExoU from *P. aeruginosa* is probably the most comprehensively described phospholipase A-like bacterial cytotoxin [106]. It exerts phospholipase A2, lysophospholipase, lipase and acyl hydrolase activities [107, 108]. Protein is injected directly in the host cell by type III secretion mechanism [109]. The bacteria deliver ExoU in its inactive form which is than activated by interaction with eukaryotic activator factor superoxide dismutase [110]. The secretion is followed by its specific localization to plasma membrane of host cells what bring it in vicinity of substrates and is essential for the enzymatic activity and toxicity of ExoU (Figure 6) [111, 106]. Furthermore, the interaction with host membrane results with its covalent modification by attachment of two ubiquitin molecules to ExoU [106]. The physiological reason for this modification still has to be elucidated but seams that reason might be protein trafficking to this localization rather than signal for its localization or degradation, as it is typically ubiquitylation. The observed secretion directly in host cells, activation in presence of eukaryotic cofactor and localization to the host cell membranes demonstrate powerful tool of *P. aeruginosa* to specifically destroy target cells but itself remain intact.

The studies of ExoU regions important for various functions have been identified; catalytic PLA2 domain with Ser142 and Asp344 [112], C-terminal region (aa 550-687) necessary for membrane localisation [111] and regions interacting with eukaryotic superoxide dismutase upon ExoU activation [63]. The increasing knowledge and interest in this potent virulence factor of *P. aeruginosa* have resulted in identification of pseudolipasin A, specific inhibitor of ExoU mediated PLA2 activity which could be a model for new virulence-based terapeutics [113].

In vivo ExoU is able to initiate inflammatory response in eukaryotes [114] although of greater importance is its rapid lyses of cell membranes [115] (Figure 6) which background remains unclear. Anyway large changes in phospholipid composition of yeast or mammalian cells and evidences about the fragmentation of yeasts vacuoles upon the intracellular localisation of ExoU stress the hydrolysis of membrane phospholipids as a factor mediating the cytotoxicity (Figure 6) [115]. Thus, the rolle of ExoU in ventilator-associated pneumonia [116, 117], pneumosepsis [118] and epithelial injuriy [119] caused by *P. aeruginosa* was confirmed [63].

However, it might be mentioned that *P. aeruginosa* PA01, as some other *P. aeruginosa* strains does not possesses a gene encoding *exoU* on the chromosome. For example, approximately 30% of *P. aeruginosa* clinical isolates encodes chromosomally *exoU* [120]. It was observed that *P. aeruginosa exoU* positive strains isolated from pneumonia patients are more virulent versus *exoU* negative strains [121]. Taken together *in vitro* and *in vivo* experiments highlight *P. aeruginosa* PLA2, ExoU, as an important factor in bacterial pathogenesis.

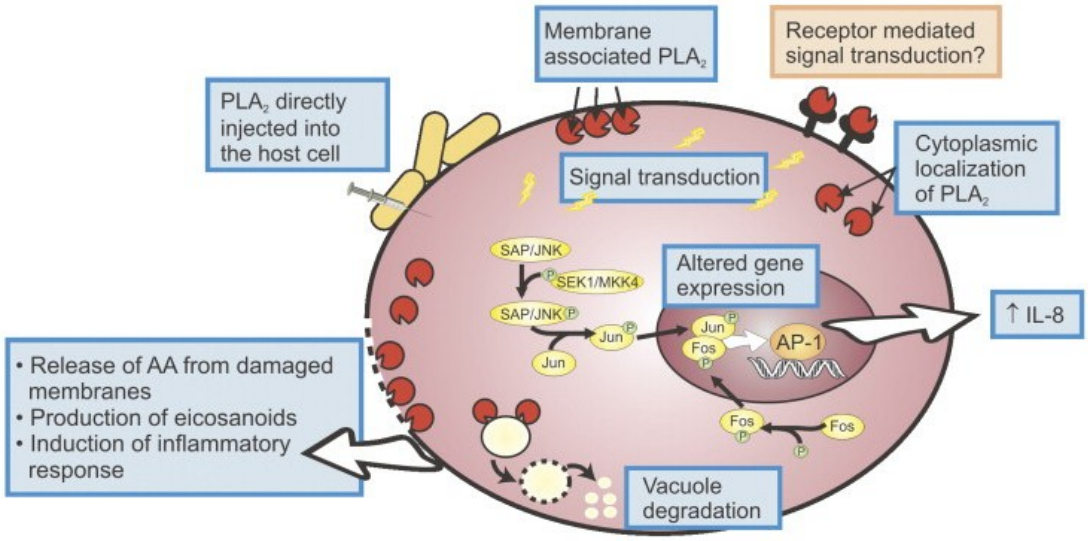


Figure 6: Demonstrated (blue windows) and putative (brown windows) effects of ExoU on eukaryotic cells. Beside above mentioned effects of ExoU on the scheme are shown ability of ExoU to alters gene expression by inducing an expression of c-Fos and c-Jun which combine in the nucleus to AP-1 that controls production of IL-8, an cytokine which activates polymorphonuclear leukocytes in the inflammatory process. The bacterial cells (in yelow) are atached to the eukariotic cell (in violet), type III secretion machinery is represented by the syringe. Adopted from Sitkiewicz *et al.* (2007) [43].

Table 3 summarise the known phospholipases A from pathogenic bacteria of which some are suggested as virulence factors and some are not. However, for those virulence factors the mechanism of action on the host is still unclear. We can notice the small number of identified phospholipases A in pathogen bacteria and even less knowledge about their function and relatedness to bacterial virulence. Therefore, this group of enzymes might represent a huge potential of bacterial virulence traits.

Table 3: Phospholipases A from pathogenic bacteria and their involvement in virulence.

Organism	Enzyme name	Virulence	Activity	Reference
Pseudomonas aeruginosa	ExoU	+	PLA2, LysoPLA, lipase	[107] [108]
Yersinia enterocolitica	YplA	+	PLA2	[87]
Streptoccocus sp.	SlaA	+	PLA2	[99]
Legionella pneumophila	PlaB	+	PLA, LysoPLA	[122]
Legionella pneumophila	PlaA	-	PLA	[123]
Legionella pneumophila	PlaC	-	LysoPLA, acyltransferase	[124]
Streptococcus sp.	SsE	+	Esterase	[125]
Helicobacter pylori	OMPLA	+	PLA	[83]
Mycobacterium tuberculosis	MPLA	-	PLA, cutinse	[126]
Mycobacterium tuberculosis	Rv3802c		PLA, thioesterase	[50]

1.4. Phospholipases from GDSL-hydrolases family

Fifteen years ago a new family of lipolytic enzymes with catalytic serine consensus motif (GDSL) distinct from most other lipolytic enzymes (GXSXG) was proposed [127]. The structures of GXSXG-hydrolases share common protein fold called α/β-hydrolase fold [128]. It is characterised by central hydrophobic sheet composed of mostly eight β-strands which are connected by α-helices [129, 128]. The glycins of GXSXG account for localisation of catalytic Ser on the top of sharp turn, so called "nucleophilic elbow". The α-helix regularly precedes and β-strand follows catalytic serine thus defining a nucleophilic elbow, probably the best conserved motif of α/β-hydrolases [128]. Therefore, significant differences in protein fold of GXSXG- and GDSL-hydrolases as a consequence of sequence motif enclosing catalytic serine and the localisation of catalytic serine relatively to the N-terminal residue were suggested [127]. In parallel with the proposed new GDSL-hydrolase family first crystal structure of its member was published, structure of esterase from *Streptomyces scabies* (SsEst) [130]. Although, the authors of SsEst have observed catalytic serine (Ser14) close to the N-terminus and they suggested a tertiary fold different from all other α/β-hydrolases they have not recognised SsEst as a GDSL-hydrolase. Short after publication of crystal structure of SsEst another two structures of GDSL-hydrolases, mammalian platelet-activating factor acylhydrolase [131] and haemagglutinin-esterase-fusion glycoprotein (HEFG) of influenca C virus [132], were published but without relating them to the GDSL-hydrolase family. The structure of rhamnogalacturonan acetylesterase (RGAH) from *Aspergillus aculeatus* published by Mølgaard *et al.* (2000) [133] had a crucial impact to the definition of GDSL-hydrolase family. Thus the authors have recognised structural homology of RGAH to SsEst and PAF AH (not to HEFG) which led to definition of three layer α/β/α-fold of GDSL-hydrolases distinct to the common α/β-hydrolase fold (Figure 7).

The α/β/α-hydrolase fold of GDSL-hydrolases, RGAH, PAF AH, SsEst, *E. coli* thioesterase/lysophospholipase/protease (TAP) [3], HEFG, reveal conserved core structure consisting of five β-strands and at least four α-helices (Figure 7). However, additional secondary structure elements inserted in canonical structure and particular difference in loops, building a substrate-binding site, inserted in canonical structure support evolutionary differentiation within a common fold [3]. Such plastic character of protein fold is well understood feature of α/β-hydrolase fold in which loops are inserted in β-sheet for accommodating various substrates [134]. Similar to α/β- fold, functional impact of the plasticity of α/β/α-fold is underlined by various substrates of GDSL-hydrolases ranging from glycoesters, arylesters, wax-like lipids, phospholipids, lysophospholipids, acyl-CoA thioesters, peptides, triacylglycerols [3, 133, 4]. The enzymes of the SGNH family originate from plants, bacteria, mammals, fungi, viruses indicating a broad phylogenetic distribution of these enzymes what furthermore support an idea of plasticity of α/β/α-fold.

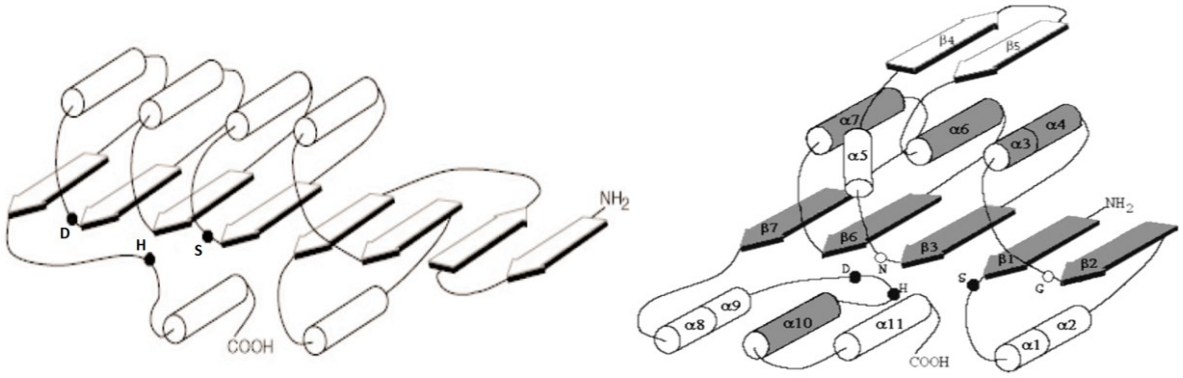


Figure 7: Topology diagrams of classical α/β-fold (left) and α/β/α-fold (right) of *A. aculeatus* RGAE. Minimal α/β/α-fold defined as a secondary structure elements conserved in SGNH-hydrolases (RGAE, SsEst, PAF AH, TAP, HEFG) is represented in gray colour. Black dots represent catalytic triad residues (Ser, His, Asp) and white dots represent conserved oxyanion hole residues (Gly, Asn). Modified figure from Mølgaard *et al.* (2000) [133].

One of the best example of plastic $\alpha/\beta/\alpha$-fold is an HEFG of influenca C virus [132] consists of three domains; domain responsible for fusion to the membrane, esterase (GDSL) domain involved in receptor inactivation and a receptor binding domain. The structure of HEFG revealed receptor domain inserted into a surface loop of esterase domain (Figure 7). Thus, esterase domain consists of amino acids 41-150 and 311-366, and receptor domain consequently corresponds to the sequence 151-310.

Furthermore, the structure of RGAH has clarified the significance of short conserved sequences for the function of GDSL-hydrolases. Thus, in the pioneer work of Upton and Buckley (1995) [127] five conserved regions (blocks I-V) were recognised suggesting Ser in block I, Asp in block III His in bloc V as a catalytic residues. In sequence homology analysis of 22 GDSL enzymes performed by Dalrymple *et al.* (1997) three conserved regions, representing blocks I, III and V (Upton and Buckley [127] numbering) were recognised [135]. The structure of RGAH [133] clearly demonstrated conservation of sequences falling in four homology blocks which correspond to blocks I, II, III and V (Figure 8) recognised by Upton and Buckley [127]. Furthermore, the structural homology analysis revealed that Ser (block I), His (block V) and Asp (block V), although in SsEst Tyr re*Plac*es functionally and structural Asp, are members of catalytic triad [133]. The catalytic role of Asp from block III proposed by Upton and Buckley, probably by analogy to GXSXG-hydrolases in which catalytic triad Asp and His are separated by 50 or more amino acids, seems to be wrong. Thus, additional feature distinguishing GDSL-hydrolases and GXSXG-hydrolases is a distance of catalytic triad His and Asp in sequence. In GDSL-hydrolases they are separated always by two amino acids and in GXSXG-hydrolases by more amino acids.

	Blocks				Accession Numbers
	I	II	III	V	Expasy / PDB
RGAE	VYLAGDSTMA	VNDAVAGRS	VIVEFGHNDGG	PIDHTHTS	Q00017 / 1DEO
SsEst	TVFFGDSYTA	ADVSCGGAL	TVGSLGGNTLG	IPWYAHPN	M57297 / 1ESC
PAF AH	VVFIGDSLVQ	LNFGIGGDS	VVVWVGTNNHG	MYDYLHLS	Q29460 / 1WAB
HEF	WIGFGDSRTD	KFRFLSGGS	KVFYEGVNWSP	EADDHHGD	P07975 / 1FLC
TAP	LLILGDSLSA	VNASISGDT	VLVELGGNDGL	QDDGIHPN	P29679 / 1JRL
	*			* *	
	S	G	N	H	HYDROLASES

Figure 8: Alignment of four homology blocks (I, II, III and V) characteristic for the GDSL-family of hydrolases: SsEst, *S. scabies* esterase; PAF AH, bovine platelet activating factor acetylhydrolase IB; TAP, *E. coli* thioesterase; RGAE, *A. aculeatus* rhamnogalacturonan acetylesterase and HEF, Influenza C virus haemagglutinin-esterase-fusion glycoprotein (HEFG). Strictly conserved residues are shaded in dark grey. The active site residues are marked by asterisks.

Furthermore, GDSL-hydrolases lack a nucleophilic elbow strongly conserved among GXSXG-hydrolases. Despite, obvious differences in consensus sequences around catalytic triads of GDSL-hydrolase and GXSXG-hydrolases, three dimensional rearrangement of catalytic triad in those two families are almost the same (Figure 9). The, conserved sequences of Block II and III enclose single Gly and Asn, respectively, which were confirmed as residues contributing to the stabilisation of substrate-enzyme complex formed during the hydrolysis. Thus, in each of the four conserved blocks a single catalytic amino acid is conserved, namely Ser (block I), Gly (block II), Asn (block III), and His (block V), leading to the proposal of new name for this family, SGNH-hydrolase family (Figure 8) [133], recently reviewed by Akoh *et al.* (2004) [4]. Today GDSL- and SGNH-hydrolase names are used synonymously in the literature.

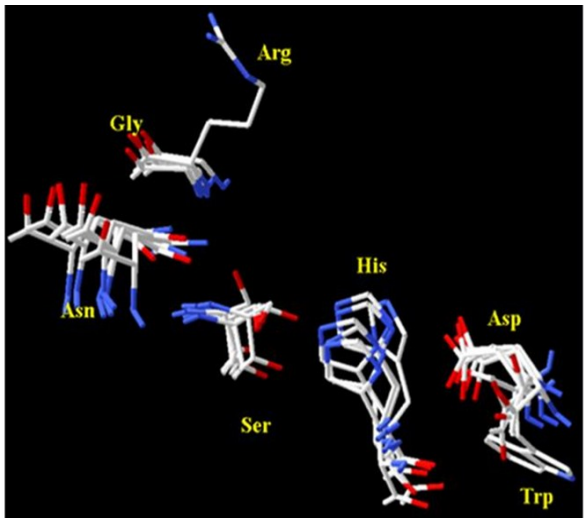


Figure 9: Topological similarity of catalytic triads and oxyanion hole residues of GDSL-hydrolases. In superposition were used structures of GDSL-hydrolases listet in Figure 8 and *Nostoc* sp. putative lipase. In the oxyanion hole of the esterase from *Nostoc sp*. Arg54 takes over the role of Gly. In the catalytic triad of the esterase from *S. scabies*, Trp280 takes over the role of Asp. Nitrogen, oxygen and carbon atoms are depicted in blue, red and gray colour respectively. Superposition was performed with SwissPdb Viewer program.

TAP from *E. coli* is the most studied GDSL-hydrolytic enzyme discovered independently from three research groups, as a thioesterase (TesA) [136], a protease (ApeA) [137] and a lysophospholipase A (PldC) [44]. Later was shown that all three enzymes are encoded by the same gene [138], and enzyme is named TAP. The particular multifunctionality of TAP led to the multiple solving of its crystal structure, as a native protein and in complexes with an inhibitor and substrate [3, 139]. Beside the TAP structures solved in solid state it was studied in solution by NMR methods [140, 141]. Furthermore, comprehensive set of kinetic data of active site point mutants and other important amino acids have reveal an details on mutifunctionality of TAP [142, 143, 144].

Thus, the typical $\alpha/\beta/\alpha$-fold and active site of GDSL-hydrolase were detected in TAP [3]. Catalytic triad of TAP is composed of Ser10, Asp154 and His157 in arrangement where side chains of Asp and His are connected by H-bond. Thus, His serves as a proton acceptor for the proton of the side chain Ser hydroxyl group. So activated Ser than perform nucleophilic attack to the carbonyl carbon atom of ester bound. Consequently, negatively charged tetrahedral substrat-enzyme intermediate is formed. This was confirmed in the structure of TAP with covalently bound inhibitor in the active site [3]. The stabilisation of intermediate occurs trough the interaction of oxyanion atom of substrate and three H-bond donors, Ser10 and Gly44 (with their backbone amid groups) and side chain nitrogen atom of Asn73. Three oxyanion hole stabilising interaction are typical for GDSL-hydrolases. In a next step catalytic triad histidine give a proton to the leaving group of substrate and deprotonate a water molecule. Thus, formed hydroxyl anion performs a nucleophilic attack to the acyl-enzyme complex resulting in braking of acyl-enzyme ester bound and releasing of acyl moiety from the active site [142]. The residual activity of TAP protein with replaced catalytic serine against alanine (S10A mutant) suggested that His deprotonates a water molecule in the active site which than directly performs a nucleophilic attack to the ester bound resulting in the hydrolysis [142]. This, GDSL-hydrolase catalytic mechanism is similar to the general mechanism of Ser-hydrolases which all have a Ser-His-Asp catalytic triad [48].

Despite an H-bond network of catalytic triad and oxyanion hole similar to serine hydrolases the active site of TAP should reveal some unique features since TAP is multifunctional enzyme. Indeed, the architecture of TAP active site indicates only one obvious gorge on the surface and it is in vicinity of active site. This site could bind an fatty acid chain of TAP substrates and the alcohol moieties may interacts with the TAP surface without specific cavities [3]. Thus, TAP can bind different esters, thioesters, lysophospholipids, arylesters or even protease substrate. NMR studies have revealed particular flexibility of loops that form the substrate binding pocket [141] what is in keeping with observed binding of wide range of substrate by TAP. Furthermore, the role of the flexible active site for TAP catalysis was confirmed by demonstrating an remarkable structural changes in one loop upon binding of octanoic acid (cleavage product of TAP substrates) in the active site [139]. This loop is called “switch loop” and it was demonstrated that Leu109 play an important role in dynamic of this loop. Thus, the mutation L109P make this loop more rigid what has direct influence on thioesterase and esterase activity of TAP which are respectively 7 and 10 fold lower in L109P mutant than in native TAP [139].

The GDSL-hydrolase family is characterised by low sequence homology outside of four conserved blocks of homology [4]. This is supporting a function of conserved regions in enzymatic catalysis what was furthermore demonstrated by structural and biochemical studies. However, the slight changes in conserved sequences were observed suggesting an existence of different subfamilies inside a large GDSL-hydrolase family. Thus, the structures of putative carbohydrate esterases from *A. thaliana* [145] and *C. acetobutylicum* [146] revealed a role of glutamine preceding catalytic serine in sequence in oxyanion hole instead of typically Gln from block III. Indeed, in these enzymes at the position of the oxyanion hole Gln in block III was found Ser residue. The, topology of other active site residues and the $\alpha/\beta/\alpha$-fold of these carbohydrate esterases are similar to canonical GDSL-hydrolases. Thus, GQSN motif in block I conserved among known carbohydrate esterases may be a signature of this GDSL-hydrolase subfamily. Furthermore, Anantharman and Aravind [147] have recently proposed a novel eukaryotic esterase family with a similar fold and catalytic triad to the GDSL-hydrolases but

novel family lack conserved oxyanion hole Gly and Asn from block II and III respectively. However, at the position of Asn from block III of canonical GDSL-hydrolases is conserved Trp or His. This novel GDSL-hydrolase family was named PC-esterase family.

Physiological function of GDSL-hydrolases cannot be generalised due to the wide range of substrates they hydrolyse. However, there are several reports about involvement of different GDSL-hydrolases in cell surface modification. Thus, degradation of pectin, a cell wall polysacharid is a probable function of RGHA [133]. Furthermore, fungal acetylxylan esterases were proposed to influence a digestibility of plant cell wall by removing acetyl group from oligoxylanesters [135]. The eukaryotic GDSL-hydrolases from PC-family were proposed to modify a cell surface polymers [147]. Additionally, HEFG, an envelope protein of Influenca C virus, has a function to destroy a eukaryotic surface exposed receptor (9-O-acetylsialic acid) after virus binding. Thus, GDSL-hydrolase may have a significant role in cell surface modifying processes.

Some bacteria express GDSL-hydrolases with phospholipase A function. LipC of *B. subtilis* is a phospholipase B that is probably involved in the degradation of the outer spore membrane during sporulation [70]. *L. pneumophila* expresses three GDSL-hydrolases with proven or putative role in virulence [148]. PlaA is secreted protein with LysoPLA activity [149], *Plac* is extracellular PLA with acyltransferase activity [124] and PlaD is extracellular PLA [150]. It is supposed that *L. pneumophila* PlaA play a role in the protection of bacteria from the toxic lysophospholipids [123]. It has been shown that *L. pneumophila* destroys protecting lung surfactant by phospholipid degradation therefore it might be that *Plac* and PlaD contribute to this process [148]. In the outer membrane of *P. aeruginosa* an esterase (EstA) was detected that belongs to the GDSL-hydrolase family [28] with side PLA activity [2]. It is required for the production of biosurfactant (Rhamnolipid) [1] with demonstrated protecting function against eukaryotic phagocytic cells [91]. Furthermore, EstA is required for formation of biofilm [1] that is common lifestyle of *P. aeruginosa* in the lungs of cystic fibrosis patients [151]. EstA is a two domain protein with its esterase domain exposed on the cell surface and C-terminal domain folded in to the outer membrane [28]. The β-barrel structure of membrane domain of EstA suggested a secretion of EstA via autotransporer mechanism, the translocation of esterase domain of EstA across the outer membrane trough the C-terminal β-barrel like channel of EstA. Although the crystal structure of full-length EstA [152] does not reveal clear evidences about a mechanism of surface exposure of EstA it is not doubtful that numerous bacterial outer membrane virulence factors exhibits similar organisation [153]. Therefore, EstA, an only GDSL-hydrolase detected in *P. aeruginosa* to date represents an interesting enzyme of *P. aeruginosa* from structural and pathophysiological point of view.

Thus, structural analysis of GDSL-hydrolases, enzymes with low overall sequence homology, revealed a sequence features which allow certain determination of new members. As a consequence today are available more than 5000 sequences of GDSL-hydrolases which represent a large pool of physiologically and biotechnologically promising hydrolytic enzymes.

1.5. Aims of this study

In the present work we have taken a broad view on the lipolytic system of *P. aeruginosa* PA01 with the purpose to determine whether this organism, like some other *gram-negative* pathogens, expresses enzymes with phospholipase A (PLA) or lysophospholipase A (LysoPLA) activities. The establishment of PLA and LysoPLA as a bacterial virulence factors is relatively new, nevertheless, to date strong evidences exist about the requirement of these enzymes for cellular invasion and colonization of pathogen [87], stimulation of the host inflammatory response [99, 87, 115] and lysis of host cells [115]. Therefore, it is feasible to speculate about a virulence role of these enzymes in *P. aeruginosa* infections. A limited number of these enzymes are described in bacteria and no one is described in *P. aeruginosa* PA01 indicating the need to explore this human opporstunistic pathogen for its potential to produce PLA and LysoPLA .

We have chosen a rational approach to attribute PLA and LysoPLA activities to particular genes. First, we have examined whether *P. aeruginosa* PA01 wild type exhibits these activities and with which cell compartments they are associated. Furthermore, a set of ten genes of *P. aeruginosa* PA01 encoding for putative PLA/LysoPLA was defined based on sequence homology search. These candidates were examined in detail by bioinformatics methods to reveal their putative enzymatic functions and sub-cellular localizations. Moreover, a gross set of experiments in *E. coli* was performed in order to get an insight on expression, detection and function of the chosen proteins. Combining all data, the most promising enzymes, namely PlaK, TesA and PlbF, were explored in details. Their biochemical characterization, purification and sub-cellular localization in *P. aeruginosa* PA01 were examined. In order to find novel virulence factors of *P. aeruginosa* PA01, and the first one belonging to the group of PLA or LysoPLA, respective *P. aeruginosa* PA01 negative mutants were tested in a *Drosophila melanogaster* virulence model.

2. Materials

2.1. Chemicals and enzymes

Used antibiotics, chemicals, medium components and enzymes in p.a. purity grade were purchased from:

Antibiotics: Gerbu (Geilberg), Serva (Heidelberg), Sigma (Deisenhofen).

Chemicals: Biomol (Hamburg), Fluka (Sternheim), Gibco BRL (Eggenstein), Merck (Darmstadt), Pharmacia (Freiburg), Roth (Karlsruhe), Sigma (Deisenhofen), Serva (Heidelberg).

Enzymes: Restriktion enzymes were obtained from MBI Fermentas (St. Leon-Rot), New England Biolabs (Schwalbach) and Roche Applied Science (Mannheim). Other enzymes were obtained from: Lysozym - Sigma (Deisenhofen), T4-DNA-Ligase, T4 DNA Polymerase - MBI Fermentas (St. Leon-Rot), *Pfu* DNA Polymerase - Stratagene (Heidelberg), Phusion DNA Polymerase – Finnzymes (Espoo, Finland), High Fidelitiy DNA polymerase - Roche Applied Science (Mannheim).

Medium components: Difco (Detroit, USA), Gibco BRL (Eggenstein), Oxoid (Wesel).

2.2. Bacterial strains and plasmids

All bacterial strains and plasmids used and created in frame of this PhD thesis are summarized in table 4 and table 5 respectively.

Table 4: Description of bacterial strains used and created in this PhD thesis.

Strain name	Genotype	Citation
Escherichia coli DH5α	*supE44* Δ(*lacZYA-argF*)*U196* (*Φ80ΔlacZM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	[154]
Escherichia coli BL21(DE3)	F⁻ *ompT hsdSB*($r_B^- m_B^-$) *gal dem* (*λIts*857 *indI Sam*7 *nin*5 *lavUV*5-T7*gene*1*)*	[155]
Escherichia coli S17.1	Ec294:: [RP4-2(Tc::Mu)(Km::Tn7)] Tp^r, Sm^r, *tra*$^+$ *recA, pro, res*	[156]
Escherichia coli TOP10	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K *rps*L(strr) *end*A1 *nup*G	Invitrogen
Escherichia coli CC118 λpir	Δ(*ara, leu*) *araD* Δ*lacX*74 *galE galK phoA*20 *thi-1 rpsE rpoB argE(Am) recA λpir* phage lysogen	[157]
Pseudomonas aeruginosa PAO1	wild type originating from Dieter Haas laboratory (Lausanne, CH)	[158]
Pseudomonas aeruginosa PASCHI	PAO1, Δ*estA*::ΩSm^r/Sp^r	[159]
Pseudomonas aeruginosa ΔplbF	PAO1, Δ*plaB*::ΩSm^r/Sp^r	this study
Pseudomonas aeruginosa ΔlipF	PAO1, Δ*lipF*::ΩSm^r/Sp^r	this study
Pseudomonas aeruginosa ΔplaB	PAO1, Δ*plaB*	this study

Table 5: Plasmids used and created in frame of this PhD thesis.

Plasmid name	Genotype	Reference
pET22b+	ColE1 PT7$_{\Phi 10}$ *pel*B Ap^r C-His$_6$-„Tag“ *lacI*^{q}	Novagen
pBBR1mcs-3	Cm^r mob *lacZα Plac* PT7 Tc^r	[160]
pBR22b	Cm^r mob *lacI*^{q} PT7$_{\phi 10}$	[161]
pSUP202	ColE1 *mob* Ap^r Cm^r Tc^r	[156]
pRK2013	ColE1 replicon with RK2 transfer region Nm-Km^r	[162]
pKNG101	*oriR6K* Stp^r *mobRK2 sacB*	[163]
PCR 2.1	*lacZα Plac* PT7$_{\phi 10}$ Km^r Ap^r *oripUC orif1*	Invitrogen
pBSL142	Ap^r, Gm^r, ColE1	[164]
pET22b-PlaK	*plaK* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pET22b-PlaKH6	*plaKH6* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pBR22b-PlaK	*plaK* gene inserted in *Nde*I/*Sac*I of pBR22b(+)	this study
pBBR-PlaK	*Xba*I/*Sac*I fragment of pET22b-PlaK inserted in pBBR1mcs-3	this study
pET22b-EstB	*estB* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pET22b-EstBH6	*estBH6* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pBR22b-EstB	*estB* gene inserted in *Nde*I/*Sac*I of pBR22b(+)	this study
pBBR-EstB	*Xba*I/*Sac*I fragment of pET22b-EstB inserted in pBBR1mcs-3	this study
pET22b-PldB	*pldB* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pET22b-PldBH6	*pldBH6* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pBR22b-PldB	*pldB* gene inserted in *Nde*I/*Sac*I of pBR22b(+)	this study
pBBR-PldB	*Xba*I/*Sac*I fragment of pET22b-PldB inserted in pBBR1mcs-3	this study
pET22b-TesA	*tesA* gene inserted in *Nde*I/*Sac*I in pET22b(+)	this study
pET22b-TesAH6	*tesAH6* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pBR22b-TesA	*tesA* gene inserted in *Nde*I/*Sac*I of pBR22b(+)	this study
pBBR-TesA	*Xba*I/*Sac*I fragment of pET22b-TesA inserted in pBBR1mcs-3	this study
pBBR-TesAH6	*Nde*I/*Sac*I fragment of pET22b-TesAH6 inserted in pBBR1mcs-3	this study
pET22b-TesA$_{S30A}$	*tesA* gene with exchanged Ser30 against Ala inserted in *Nde*I/*Sac*I in pET22b(+)	this study
pET22b-TesA$_{D177A}$	*tesA* gene with exchanged Asp177 against Ala inserted in *Nde*I/*Sac*I in pET22b(+)	this study
pET22b-TesA$_{H180A}$	*tesA* gene with exchanged His180 against Ala inserted in *Nde*I/*Sac*I in pET22b(+)	this study
pET22b-PlaB	*plaB* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pET22b-PlaBH6	*plaBH6* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pBR22b-PlaB	*plaB* gene inserted in *Nde*I/*Sac*I of pBR22b(+)	this study
pBBR-PlaB	*Xba*I/*Sac*I fragment of pET22b-PlaB inserted in pBBR1mcs-3	this study
pET22b-PlbF	*plbF* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pET22b-PlbFH6	*plbFH6* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pBR22b-PlbF	*plbF* gene inserted in *Nde*I/*Sac*I of pBR22b(+)	this study
pBBR-PlbF	*Xba*I/*Sac*I fragment of pET22b-PlbF inserted in pBBR1mcs-3	this study
pBBR-PlbFH6	*Xba*I/*Sac*I fragment of pET22b-PlbFH6 inserted in	this study

	pBBR1mcs-3	
pET22b-PlpD	*plpD* gene inserted in *Nde*I/*Sac*I of pET22b(+)	
pET22b-PlpDH6	*plpDH6* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pBR22b-PlpD	*plpD* gene inserted in *Nde*I/*Sac*I of pBR22b(+)	this study
pBBR-PlpD	*Xba*I/*Sac*I fragment of pET22b-PlpD inserted in pBBR1mcs-3	this study
pET22b-EstC	*estC* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pET22b-EstCH6	*estCH6* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pBR22b-EstC	*estC* gene inserted in *Nde*I/*Sac*I of pBR22b(+)	this study
pBBR-EstC	*Xba*I/*Sac*I fragment of pET22b-EstC inserted in pBBR1mcs-3	this study
pET22b-PldC	*pldC* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pET22b-PldCH6	*pldCH6* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pBR22b-PldC	*pldC* gene inserted in *Nde*I/*Sac*I of pBR22b(+)	this study
pBBR-PldC	*Xba*I/*Sac*I fragment of pET22b-PldC inserted in pBBR1mcs-3	this study
pET22b-LipF	*lipF* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pET22b-LipFH6	*lipFH6* gene inserted in *Nde*I/*Sac*I of pET22b(+)	this study
pBR22b-LipF	*lipF* gene inserted in *Nde*I/*Sac*I of pBR22b(+)	this study
pBBR-LipF	*Xba*I/*Sac*I fragment of pET22b-LipF inserted in pBBR1mcs-3	this study
pSUP-Δ*lipF*	pSUP202 containing DNA element consists of 1000bp upstream region of *lipF* followed by Gm-Ω cassette (*Mlu*I/*Mlu*I fragment of pBSL142) and 1000bp downstream region of *lipF* gene	this study
pSUP-Δ*plbF*	pSUP202 containing DNA element consists of 1000bp upstream region of *plbF* followed by Gm-Ω cassette (*Mlu*I/*Mlu*I fragment of pBSL142) and 1000bp downstream region of *plbF* gene	this study
pCR2.1-Δ*plaB*	Δ*plaB* fragment consists of 547bp upstream region followed by 433bp downstream regions of *plaB* gene inserted in pCR2.1	this study
pKNG-Δ*plaB*	pKNG101 with inserted *Nsi*I/*Bam*HI fragment of PCR2.1-Δ*plaB*	this study

2.3. Oligonucleotides

All oligonucleotides (Table 6) used in PCR were purchased from Eurofins MWG Operon (Ebersberg) as high purified salt free compounds delivered in lyophilized form. Prior to using primers were dissolved in sterile A. dest. yielding 100 pmol/µl solutions. Resulting primer solutions were stored at -20°C.

Table 6: Oligonucleotides used in PCR.

Name	DNA Sequence (5'-3')	Features[a]
PA4921Up	AAACATATGCGCACCCGTCTC	*Nde*I
PA4921Down	TAAGAGCTCTAACTCGAGGCGCGCGTAGCGTGC	*Sac*I
PA4921DownHis6	ATGAGCTCGTGGTGGTGGTGGTGGTGCGCGCGTAGCGTGC	*Sac*I, His_6
PA2856Up	TAACATATGCGTGCATTGCTG	*Nde*I

PA2856Down	TAAGAGCTCTAACTCGAGAAGCAGCGGTTTCAG	*Sac*I
PA2856DownHis6	ATGAGCTCGTGGTGGTGGTGGTGGTGAAGGAGCGGTTTCAG	*Sac*I, His_6
PA2856UpS30A[b]	GTGGGCGAT**GCT**ATCAGCGCC	Ala
PA2856DownS30A[b]	CACCCGCAT**CGA**TAGTCGCGG	Ala
PA2856UpD177A[b]	ATGCAGGCG**GCT**GGCATCCAT	Ala
PA2856DownD177A[b]	TACGTCCGC**CGA**CCGTAGGTA	Ala
PA2856UpH180A[b]	GATGGCATC**GCT**CCGGCGCTC	Ala
PA2856DownH180A[b]	CTACCGTAG**CGA**GGCCGCGAG	Ala
PA0844Up	AAACATATGACCGAAAACTGG	*Nde*I
PA0844Down	ATGAGCTCTCAGGTCGCTGCGATGTC	*Sac*I
PA0844Down His6	TCAGAGCTCGTGGTGGTGGTGGTGGTGGGTCGCTGCGATGTC	*Sac*I, His_6
PA1047Up	AAACATATGCCCCGACCACTT	*Nde*I
PA1047Down	ATGAGCTCTTACAGACACTCCCCCGC	*Sac*I
PA1047Down His6	TCAGAGCTCGTGGTGGTGGTGGTGGTGCAGACACTCCCCCGC	*Sac*I, His_6
PA3487Up	AAATCTAGAATGTTGCAGAAGAAG	*Nde*I
PA3487Down	ATGAGCTCTCATGTGCGATGCTTTCC	*Sac*I
PA3487Down His6	TCAGAGCTCGTGGTGGTGGTGGTGGTGTGTGCGATGCTTTCC	*Sac*I, His_6
PA3628Up	AAACATATGAAGGATTCCCTC	*Nde*I
PA3628Down	ATGAGCTCTCAACCGTTGAGAACCCC	*Sac*I
PA3628Down His6	TCAGAGCTCGTGGTGGTGGTGGTGGTGACCGTTGAGAACCCC	*Sac*I, His_6
PA2949Up	AAACATATGAAACGATTCCTC	*Nde*I
PA2949Down	ATGAGCTCTCAGCGACCGGCCAC	*Sac*I
PA2949DownHis6	TCAGAGCTCGTGGTGGTGGTGGTGGTGGCGACCGGCCAC	*Sac*I, His_6
PA2098Up	AAACATATGTCGCCGCCGCAA	*Nde*I
PA2098Down	ATGAGCTCTCAGGATGTTCTGCA	*Sac*I
PA2098DownHis6	TCAGAGCTCGTGGTGGTGGTGGTGGTGGGATGTTCTGCA	*Sac*I, His_6
PA3339Up	AAACATATGCGCCGCCTGCTG	*Nde*I
PA3339Down	ATGAGCTCTCAGAAGTTCTGCCC	*Sac*I
PA3339DownHis6	TCAGAGCTCGTGGTGGTGGTGGTGGTGGAAGTTCTGCCC	*Sac*I, His_6
PA2155Up	AAACATATGAACTTCCCCTGG	*Nde*I
PA2155Down	ATGAGCTCTCATGGGTTGCCCCG	*Sac*I
PA2155DownHis6	TCAGAGCTCGTGGTGGTGGTGGTGGTGTGGGTTGCCCCG	*Sac*I, His_6
PA4339Up	AAACATATGCTGCTGGCCATC	*Nde*I
PA4339Down	ATGAGCTCCTAGCGCCGGCGGTC	*Sac*I
PA4339DownHis6	TCAGAGCTCGTGGTGGTGGTGGTGGTGGCGCCGGCGGTC	*Sac*I, His_6
PA2927Up	AAACATATGCCCCGTTCGATC	*Nde*I
PA2927Down	ATGAGCTCCTATCAGGGGTTCCTGAA	*Sac*I
PA2927DownHis6	TCAGAGCTCGTGGTGGTGGTGGTGGTGTCAGGGGTTCCTGAA	*Sac*I, His_6
ΔPA2949UpU	AAAAAACGCGTGCCGCCCTGGCCCTGGCCGA	*Mlu*I
ΔPA2949UpD	AAAAAGAGCTCAAAAATCTAGAGGGTGTCCGAAGGCAACAGG	*Sac*I, *Xba*I
ΔPA2949DownU	AAAAATCTAGAAAAAAACGCGTAAACGCGAACCGGCGCCTGG	*Xba*I, *Mlu*I
ΔPA2949DownD	AAAAAGAGCTCGGCGGCCTTCGAGAAGTTCG	*Sac*I
ΔPA4921UpU	AAACGCGTGTGCCGGCCTAATCCTTT	*Mlu*I
ΔPA4921UpD	AAGAGCTCAATCTAGAGGCTGGCCTTCGCAGGGATA	*Sac*I, XbaI
ΔPA4921DownU	ATCTAGATTACGCGTGCCCGGCGCGCGCCCATG	*Xba*I, *Mlu*I
ΔPA4921DownD	AAGAGCTCCAGGCTCTGCGGGATGAT	*Sac*I
ΔPA2927UpU	CGAAGTGCTGAAAGTCATGC	-

ΔPA2927UpD	CCGGGCGCCGATTCACATGTCCGGCATCCT	-
ΔPA2927DownU	AGGATGCCGGACATGTGAATCGGCGCCCGG	-
ΔPA2927DownD	GCGTTGTACCGTCGCTATG	-

[a]The engineered restriction sites and the sequence encodes His6 peptide are underlined and double underlined as is shown in features column.

[b]The sequence corresponding to the mutated codons are shown in bold.

2.4. Culture growth media

LB medium	10 g/l Tryptone, 10 g/l NaCl, 5 g/l Yeast extract pH = 7 was adjusted with the NaOH.
LB agar medium	10 g/l Tryptone, 10 g/l NaCl, 5 g/l Yeast extract, 15 g/l Agar
PPGAS medium	5 g/l Glucose, 10 g/l Peptone, 1.08 g/l NH_4Cl, 1.48 g/l KCl, 14.6 g/l Tris-HCl, 0.36 g/l $MgSO_4$ x $7H_2O$ pH was adjusted at 7.2 with the HCl
MME medium	10 g/l $K_{2\ h}PO_4$, 3.5 g/l $Na(NH_4)HPO_4$, 2 g/l glucose, 0.2 g/l $MgSO_4$ x 7 H_2O pH was adjusted at 7.0 with NaOH
M9 medium	solution 1: 40 g/l glucose solution 2: 25 g/l $MgSO_4$ x $7H_2O$ solution 3: 2 g/l $CaCl_2$ solution 4: 70 g/l $Na_{2\ h}PO_4$ x 2 h_2O, 30 g/l KH_2PO_4, 5 g/l NaCl, 10 g/l NH_4Cl Each solution was sterilized separately. The 1 l of medium was prepared mixing 100 ml solution 1, 10 ml solution 2, 10 ml solution 3 and 100 ml solution 4 with A. dest.
α-Complementation Agar	4 ml IPTG (100 mM in 70% (v/v) etanol) 16 ml X-Gal (2% (w/v) in DMF) 100 ml LB agar
TSB medium	15 g/l Tryptone 5 g/l Soytone (Papaic Digest of Soybean Meal) 5 g/l NaCl
TB medium	Solution 1: 13.3 g/l Tryptone, 26.6 g/l Yeast extract, 0.44% (v/v) Glycerol Solution 2: 0.17 M KH_2PO_4, 0.72 M $K_{2\ h}PO_4$ Both solutions were sterilized separately and the medium was prepared by mixing 900 ml solution 1 with 100 ml solution 2.

AI medium	Solution 1: 2% (w/v) Lactose, 10% (w/v) Glucose Solution 2: TB medium Both solutions were sterilized separately and the medium was prepared by mixing 20 ml of solution 1 and 980 ml of solution 2.
Buffered Agar medium	10 mM Tris-HCl buffer (pH 7.6), 8 mM $MgSO_4$, 1 mM Na-PO_4 buffer (pH 7.6), 15 g/l Agar
LB agar tributyrin plates	solution 1: 15 ml tributyrine, 1.5 g Gummi Arabicum, 30 ml A. dest. solution 2: LB agar medium The solution 1 was homogenized with the ultrasound pin and added to the 1000 ml of solution 2.
Egg Yolk LB agar medium	800 ml LB agar medium, 200 ml A. dest., 15 g EggYolk, 5 g Taurocholic acid and 2.22 g $CaCl_2$ Mix Egg Yolk, Taurocholic acid and $CaCl_2$ with water, sterilize by autoclaving it for 7 min and add to the melted LB agar medium.
Skim Milk LB agar medium	solution 1: 300 g/l Skim Milk in H_2O solution 2: 100 g/l Lactose in H_2O solution 3: LB agar medium The 100 ml of solutions 1 and 2 separately sterilized by autoclaving them for 7 min were mix with 800 ml of solution 3.

2.5. Buffers and Solution

Tris-HCl buffer	Tris-HCl base, 100mM pH was adjusted by titration with HCl
PBS buffer	11.247 g/l $Na_{2\,h}PO_4 \cdot 2\ h_2O$ 1.59 g/l KH_2PO_4 3.975 g/l NaCl Adjust the pH to 7.4 with H_3PO_4.
MES buffer	3.904 g/l MES Adjust the pH to 6.5 with NaOH.
TBS buffer	3.003 g/l Tris-HCl base 58.466 g/l NaCl 0.112 g/l KCl Adjust the pH to 8.0 with HCl solution

TBST buffer	2 ml Tween 20 998 ml TBS
Dunn Carbonate buffer	Solution 1: 0.318 g/l Na_2CO_3, 0.84 g/l $NaHCO_3$, Solution 2: Methanol Mix 100 ml solution 1 and 200 ml solution 2 with water in order to prepare 1000 ml of buffer.
Blocking reagent	30 g/l skim milk powder in TBST
Bradford reagent	100 mg/l Coomassie Brilliant Blue G-250 50 ml/l ethanol 95% (v/v) 100 ml/l phosphoric acid 85% (w/v) The solution was stirred until the dye has completely dissolved and filtered through Whatman #1 paper just before use.
Coomassie staining sol.	100 g/l Ammonium sulphate, 1% (v/v) Phosphoric acid, 0.1% (w/v) Coomassie Brilliant Blue G-250, 20% (v/v) Methanol
Coomassie distaining sol.	40% (v/v) Ethanol, 10% (v/v) Acetic acid
DNA electrophoresis buf.	89 mM Tris-HCl, 89 mM boric acid, 2.5 mM Na_2EDTA
DNA loading buffer (5x)	100 mM Na_2EDTA, 43% (v/v) Glycerol, 0.5% (w/v) Bromphenol blue
SDS electrophoresis buf.	25 mM Tris-HCl, 0.129 M glycine, 0.1% (v/v) SDS
SDS loading buffer	10% glycerol, 0.2% (w/v) SDS, 0.125 M Tris-HCl (pH = 6.8), 0.1% (w/v) Bromphenol blue, 2% (v/v) β-Mercaptoethanol
TMF buffer	100 mM $CaCl_2$, 50 mM $RbCl_2$, 40 mM $MnCl_2$
Equilibration buffer	50 mM KH_2PO_4, 5 mM Tris-HCl, 300 mM NaCl, pH = 8
Washing buffer	50 mM KH_2PO_4, 5 mM Tris-HCl, 50 mM imidazole, 300 mM NaCl, pH = 8
Elution buffer	250 mM imidazole, 50 mM KH_2PO_4, 5 mM Tris-HCl, 300 mM NaCl, pH = 8

2.6. Molecular weight standards

DNA standards: 1kbp DNA Ladder (Invitrogen)
: GeneRuler 1 kb DNA Ladder (Fermentas)
: HyperLadder I (Bioline)

Protein standards: PageRuler prestained protein ladders (Fermentas)
: PageRuler unstained protein ladders (Fermentas)
: Precision plus kaleidoscope protein ladders (BioRad)
: Kaleidoscope protein standards (Bio Rad)

2.7. Kits

InnuPREP Plasmid Mini Kit (Analytik Jena, Jena)
Plasmid DNA isolation Kit (OLS Omni Life Science GmbH & Co, Hamburg)
HiSpeed Plasmid Midi Kit (Qiagen, Hilden)
Omni-pure-OLS DNA extraction Kit (OLS Omni Life Science GmbH & Co, Hamburg)
DNAeasy Tissue Kit (Qiagen, Hilden)
ECL Western blotting detection Kit (Amersham Bioscience, Freiburg)
Perfectprep Gel cleanup Kit (Eppendorf, Hamburg)
TOPO TA Cloning Kit with pCR2.1-TOPO vector (Invitrogen, Karlsruhe)
QuantiTech SYBR Green RT-PCR Kit (Qiagen, Hilden)
Protease Colorimetric Detection Kit (Sigma, Deisenhofen)
NEFA-HR(2) Kit (Wako chemicals GmbH, Neuss)
sPLA2 Assay Kit (Cayman chemicals, Tallinn)

3. Methods

3.1. Growth conditions and storage of bacteria

3.1.1. Cultivation of *E. coli* strains

E. coli strains DH5α, TOP10, CC118 λpir and S17.1 where routinely grown at 37°C in LB medium or on the LB agar plates supplemented with antibiotics where required (Table 7). Optical density (OD) of bacterial cultures was measured at 580nm and it was expressed in "unit of cells" (U_{Cell}) where 1 U_{Cell} is equivalent to the measured OD_{580nm} of 1.

Blue-white screening of recombinant *E. coli* clones was performed on the α-complementation agar plates. Routinely cloning was performed with the *E. coli* DH5α and TOP10. Broad host range plasmids were conjugationally transferred by biparental mating between *E. coli* S17.1 and *P. aeruginosa* strains. The triparental mating was done using the *E. coli* CC118 λpir as a donor, *E. coli* DH5α carrying the plasmid pRK2013 as a helper strain and the *P. aeruginosa* strains as acceptors.

Table 7: Used antibiotics and the solvent used to prepare stock solutions. Final concentration represents a concentration of antibiotics in the growth media.

Antibiotic	Final concentration (μg/ml)		Solvent
Ampicillin	100	-	Ethanol 70% (v/v)
Chloramphenicol	50	300	Ethanol 70% (v/v)
Tetracyclin	10	100	Ethanol 70% (v/v)
Gentamycin	10	30	Water
Spectinomycin	100	100	Water
Streptemycin	-	100	Water
Irgasan	-	25	Water
Carbenicillin	-	600	Water
	E. coli	*P. aeruginosa*	

Heterologous overexpression was carried out using the *E. coli* BL21(DE3). The expression strain was grown over night at 37°C in LB medium supplement with respective antibiotic (Table 7). Overnight culture was used as inoculums for expression culture. Initial OD_{580nm} of expression culture was adjusted to 0.05 in LB medium supplement with antibiotic and 0.4% (w/v) glucose. The culture was grown until it reaches logarithmic phase, OD_{580nm} = 0.5–0.8 and at that point the overexpression was induced with 0.4 mM IPTG final concentration (stock solution: 100 mM IPTG in ethanol (70% v/v)).

The overexpression in "autoinduction medium" was performed by inoculating the expression culture at OD_{580nm} = 0.05 and growing the culture at least for 16 h. During that time spontaneous induction takes place resulting with the overexpression of target protein.

The heterologous expression under the control of Lac promoter (*Plac*) was carried out in *E. coli* DH5α using the LB medium and standard growth conditions.

3.1.2. Cultivation of *P. aeruginosa* strains

The *P. aeruginosa* strains were grown in LB medium at 37°C where otherwise is not stated. The antibiotics present in the medium to maintaining the plasmids were used according the table 7.

3.1.3. Storage of bacteria

The liquid cultures were mixed with 8% (v/v) DMSO and freeze at -80°C for longer storage period. The *E. coli* strains were kept at 4°C on the Agar plates for maximum one week. The *P. aeruginosa* strains were used from Agar plates not older than 2 days.

3.2. Isolation of nucleic acids

3.2.1. Isolation of plasmid DNA

Purification of plasmid DNA from *E. coli* and *P. aeruginosa* cultures was performed according to the standard procedure for the "HiSpeed Midi Plasmid Purification Kit", "InnuPREP Plasmid Mini Kit" and "Plasmid DNA isolation Kit" recommended by the supplier.

3.2.2. Isolation of genomic DNA

The genomic DNA of *P. aeruginosa* and *E. coli* were isolated with the "DNAeasy Tissue Kit" according the supplier recommendation.

3.3. Gel electrophoresis of nucleic acids

The agarose gel electrophoresis was used for the separation and quantification of DNA according the Sambrook (2001) [165]. In short, standard method was performed using the 0.5 fold TBE buffer and ca. 0.5 μg/ml ethidium bromide present in the gel during the DNA migration. For the elution of DNA out of the agarose gels the "Perfectprep Gel Cleanup kit" was used according to the supplied manual.

3.4. *In vitro* recombination of DNA

The endonucleolytic hydrolysis of plasmids and the PCR products by restriction endonucleases, the dephosphorilation and blunting of DNA fragments and ligation were performed as recommended by the supplier.

3.5. Transformation of bacteria with plasmid

3.5.1. Chemical transformation

Preparing and transformation of *E. coli* chemo-competent cells were performed according the Hanahan (1983) [166].

3.5.2. Electroporation of *P. aeruginosa*

For the preparation of electrocompetent cells *P. aeruginosa* cultures were inoculate in LB medium (OD_{580nm} = 0.01) using the overnight culture and grown overnight at 37°C. The overnight culture was incubated on ice for 10 minutes and diluted with sterile water (1.5 fold volume of culture) following with the centrifugation for 10 min at 4000*g* and 4°C. The cells were resuspended in 300 mM sucrose solution and centrifuged for 10 min at 4000*g* and 4°C. Three sucrose washing steps with 2.5x, 1x and 0.5x volume of culture were performed. Finally, the cells were resuspended in required volume of sucrose solution leading to the end OD_{580nm} of 50. The cells were stored in 130 μl aliquots as described (3.1.3).

The electroporation was performed in 2 mm electroporation cuvettes by adding the 20–30 ng of plasmid solution (salt free) to the one aliquot of electroporation cells. The content was briefly

vortexed and added in the precooled electroporation cuvette. The electroporation was carried out using the program EC2 (MicroPulser) and immediately after the pulse 1 ml of LB medium supplement with 0.4% (v/v) glucose to the cells was added. The culture was transferred in the 5 ml glass tube and the growth for 2 h at 37°C under aeration conditions following with the selection on the selective antibiotic LB agar plates (overnight at 37°C).

3.5.3. Conjugation of bacteria

Conjugation was performed in biparental and triparental spot mating. By the biparental mating the broad host range plasmids were conjugationally transferred between donor strain (*E. coli* S17.1) and the acceptor strain (*P. aeruginosa*). The donor strain in logarithmic phase and the overnight culture of acceptor strain incubated for 2 h at 43°C were separately centrifuged and washed with the LB medium. The donor and acceptor strain were mixed in volume ratio 2 to 1 respectively and spotted to the LB agar plate. The conjugation was performed overnight at 37°C. The conjugants were selected on the LB agar plate supplement with 25 μg/ml Irgasan and the respective antibiotic. The triparental mating was done using the *E. coli* CC118 λpir as a donor, *E. coli* DH5α with pRK2013 plasmid as a helper and *P. aeruginosa* as an acceptor strain. The same volumes of the donor and acceptor strains in logarithmic phase were centrifuged, washed with the LB medium and spotted to the LB agar plate. After the incubation for 2 h at 37°C *P. aeruginosa* cells previously incubated for 2 h at 43°C were spotted on the formed helper-donor spot. The plates were incubated for at least 6h at 37°C and the conjugants were selected on the LB agar plate supplement with 25 μg/ml Irgasan and the respective antibiotic.

3.6. Polymerase chain reaction

The DNA fragments were amplified by polymerase chain reaction (PCR) with chromosomal DNA of *P. aeruginosa* PAO1 and *E. coli* DH5α as a template [11]. The *Pfu* or Phusion DNA polymerases were used according the supplier recommendation. PCR samples contain 1 pmol/μl of each primer, 1x reaction buffer, 3% DMSO (v/v), 50 μM of each deoxynucleoside triphosphate, 2% (v/v) of template and 0.05 *U*/μl *Pfu* or 0.02 *U*/μl Phusion DNA polymerase. All PCR were performed in gradient mode using the Mastercycler gradient. The colony PCR was performed using the 1 μl of cells (~5 U_{Cell}/ml) in stationary growth phase resuspendate in TE buffer and incubated for 5 min at 99°C as a template. The applied PCR programs are summarized in Table 8. The DNA fragments of desire were separate from the unspecific DNA products by means of the agarose gel electrophoresis and they were purified with the "Perfectprep Gel cleanup Kit". When the unspecific DNA fragments were absent PCR products were purified with the "Omni-pure-OLS DNA extraction Kit".

Table 8: PCR programs applied for PCR performed with the *Pfu* and Phusion DNA Polymerase.

Step*	*Pfu*		Phusion	
	Time / s	T / °C	Time / s	T / °C
1. Initial denaturation	300	98	30	98
2. Denaturation	50	98	10	98
3. Annealing	50	$(T_m-5) \pm 2$	30	$T_m + 3$
4. Elongation	t1	72	t2	72
5. Final extension	300	72	300	72

* The steps 2, 3, 4 were repeated 15 to 35 times; T_m = the lower melting temperature of two primers; t1 = 60 x (DNA length (bp) / 500); t2 = 60 x (DNA length (bp) / 3000)

3.6.1. Site directed mutagenesis

PCR based QuickChange site directed mutagenesis method was applied to introduce a specific amino acid mutations in to the protein sequence. This method is based on the PCR amplification of complete plasmid using the two complementary oligonucleotide primers containing the mutation of interest. After the Incorporation of the oligonucleotide primers a plasmid with specific mutation is generated. Following step involves hydrolysation of DNA template by *Dpn*I endonuclease which is active only on methylated DNA. After the bacterial cell transformation with *Dpn*I treated PCR product the clones with potential mutation should be selected and plasmid should be sequenced in order to confirm presence of desirable mutation.

The method was used according the QuikChange Site-Directed Mutagenesis Kit (Stratagene) instruction manual. PCR reactions were performed in total volume of 50 μl with 50 ng of plasmid pET22(b)-TesA and PA2856UpS30A/PA2856DownS30A, PA2856UpD177A/PA2856DownD177A and PA2856UpH180A/PA2856DownH180A oligonucleotide primer pares to introduce S30A, D177A and H180A single mutations respectively. PCR program was adopted from instruction manual adjusting the amplification time to the 6 min with the respect to the plasmid length of ca. 6 kbp. The *E. coli* DH5α were transformed with 5 μl aliquots and positive clones were subjected to the sequencing. The clones with the single mutation of interest were subcloned to the pET22(b) vector using the *Sac*I and *Nde*I endonuclease restriction sites. This step is necessary in order to avoid mutations in other vital elements of the plasmid that could lead to the loose of expression.

3.7. DNA sequencing

The plasmids and PCR products were sequenced using the service of the company Sequiserve (Vaterstettten).

3.8. Construction of *lipF* and *plbF* deficient strains of *P. aeruginosa* PAO1

The method employing pSUP202 shuttle vector was used to introduce gene deletion-insertion mutation in to the chromosome of *P. aeruginosa* PA01. The corresponding 1000bp long upstream and downstream regions of genes were amplified by means of PCR and used for gene deletion. To create *lipF* deletion-insertion mutant upstream and downstream regions were amplified using the ΔPA4921UpU/ΔPA4921UpD and ΔPA4921DownU/DPA4921DownD primer pars respectively. To create *plbF* deletion-insertion mutant upstream and downstream regions were amplified using the DPA2949UpU/ΔPA2949UpD and ΔPA2949DownU/ΔPA2949DownD primer pars respectively. The gentamycin resistance gene was obtained hydrolyzing the plasmid pBSL142 with the *Mlu*I endonuclease. The gentamycin resistance gene, upstream and downstream regions were cloned in gene encodes for tetracycline resistance of the pSUP202 using the specific restriction sites. By this procedure was created recombination element consists of upstream region followed by gentamycin resistance gene and downstream region.

The shuttle vector was transformed by biparental conjugation to the *P. aeruginosa* PAO1. After the single homologues recombination takes place complete plasmid will be integrated to the chromosome what should introduce gentamycin, chloramphenicole and β-lactam resistance to the *P. aeruginosa* PA01. The recombinants which were resistant to Gm, Cm and Cb (single antibiotic was used on the LB agar plates) were grown overnight in liquid LB medium supplement with Gm. New

cultures were inoculated in LB Gm medium using the overnight cultures and the procedure was repeated at least for five cycles. The second homologues recombination on the opposite recombination site should result in lost of the shuttle vector sequence out of the chromosome and re*Plac*ement of the gene of desire against the gentamycine resistance gene. In order to select deletion-insertion mutants each clone was inoculated to the Gm, Cm and Cb LB agar plates. Clones being resistant to the Gm and susceptible to the Cb and Cm were subjected for the PCR test to confirm positive deletion-insertion mutation.

3.9. Construction of *plaB* deficient strain of *P. aeruginosa* PAO1

The method employing pKNG101 shuttle vector was used to introduce gene deletion mutation into the chromosome of *P. aeruginosa* PA01. The corresponding upstream and downstream regions of *plbF* genes spanning 547 bp and 433 bp, respectively, were amplified by means of PCR using the ΔPA2927UpU/ΔPA2927UpD and ΔPA2927DownU/ΔPA2927DownD primer pars respectively. On the primers ΔPA2927UpD and ΔPA2927DownU were introduced 12bp long homologues regions of the downstream and upstream fragments, respectively. This approach allows performing overlap PCR using the upstream and downstream fragments as a template and ΔPA2927UpU/ΔPA2927DownD primer par. The resulting PCR product amplified using the Hifi DNA polymerase (Roche) was cloned in PCR2.1 vector according the manufacture instructions. The recombination element was subcloned to the pKNG101 shuttle vector using the *Nsi*I and *Bam*HI endonuclease sites.

The shuttle vector was transformed by triparental conjugation to the *P. aeruginosa* PAO1. After the single homologues recombination take place complete plasmid should be integrated to the chromosome what introduce streptomycin resistance to the *P. aeruginosa* PA01. Additionally pKNG101 vector encodes the levansucrase gene from *B. subtilis* which is responsible for the synthesis of toxic levan in presence of sacharose [167]. The recombinants that were able to grow on the LB agar plates supplemented with Sm (2 mg/ml final concentration) were grown on the LB agar plates supplement with sacharose (5% w/v) at room temperature for 1-2 days. The clones that have lost Sm resistance but are still able to grow in presance of sacharose were tested for positive deletion mutation by means of PCR.

3.10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Discontinuoes sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS PAGE) method was performed as described by Laemmli (1970) [168].

Table 9: Components of the collection (5%) and stacking (12% - 16%) gels.

Compounds	Percentage of polyacrylamide gel		
	5%*	12%	14%
Acrylamide mixture	0.83 ml	4.00 ml	4.67 ml
Tris-HCl (1.5 M, pH = 8.8)	1.25 ml	2.50 ml	2.50 ml
H_2O	2.77 ml	3.35 ml	2.68 ml
SDS (10 % (w/v))	50 μl	100 μl	100 μl
APS (10 % 10 % (w/v))	50 μl	100 μl	100 μl
TEMED	10 μl	10 μl	10 μl

* Collecting gel (5%) was prepared with 0.5 M Tris-HCl (pH = 6.8) instead of 1,5 M Tris-HCl (pH = 8.8)

Stacking gel (5%) and separating gels (12% or 14%) were prepared using a standard procedure (table 9). Protein samples were mixed with SDS loading buffer (17% v/v) and incubated for 10 min at 95°C. Electrophoresis was performed using the BioRad (mini Protean 3 system) and SDS electrophoresis buffer. Gel was stained with Coomassie staining solution followed by discolouring with the Coomassie distaining solution.

3.11. Enzyme activity assays

Quantitative liquid assays were carried out spectrophotometrically measuring the change of absorbance due to the change of concentration of substrate or product upon the enzymatic activity. The enzyme activity was calculated according to Equation 1 and the activity was expressed in units of enzyme activity (*U*). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyse 1 μmol of a substrate in one minute under the standard conditions. The assays were carried out in cuvettes (500 μl – 3 ml) or in the 96-well microplates (100 μl – 250 μl). In continues assays performed in 96-well plates, using the microplate reader SpectraMax 250 (Molecular Devices Corp.) the data that show linear increase of absorbance with the time were used in calculations. The correction for the light path length in 96-well plates was calculated according the equation 2.

$$U/\text{ml} = \frac{A_\lambda \cdot V_{total}}{\varepsilon \cdot V_{enzyme} \cdot t \cdot l} \qquad \textbf{(Equation 1)}$$

$$l/\text{cm} = 3{,}484 \cdot 10^{-3} \cdot V_{total}/\mu\text{l} \qquad \textbf{(Equation 2)}$$

A_λ	absorbance on specific wave length
V_{total}	volume of reaction mixture (μl)
ε	molar extinction coefficient ($\text{mmol}^{-1} \cdot \text{dm}^3 \cdot \text{cm}^{-1}$)
V_{enzyme}	volume of enzyme sample used in reaction (μl)
t	time (min)
l	light path length (cm)

To avoid falsification of the results due to the spontaneous hydrolysis of the substrate "blank" sample was treated like the enzyme samples and this value was subtracted from the measured enzyme samples. Depending upon the nature of experiment the LB medium or the buffer in which were resuspendete cells or dissolved pure protein was used as the "blank" sample.

The activity assays were often used to demonstrate whether cells expressing certain enzyme differ in hydrolysis rate comparing to the cells which do not expresses enzyme. Therefore, the activities measured with cell lysates or isolated cell compartments were expressed as a difference of absorbance measured at two time points (equation 3). In one experiment (performed under the same conditions) the same amount of cells which express and which do not express target enzyme were used therefore the values (ΔA_{xxxnm}) were comparable in one experiment. However, the amount of cells and the time interval between absorbance measurmets should be noticed by comparing two distinct experiments. This is due to the fact that functions and the optimal expression conditions for

all enzymes studied in this work were unknown. Therefore, the parameters in enzymatic activity measurements (cell amount and time for performing assay) were adjusted for each enzyme and substrate individually.

$$\Delta A_{xxxnm} = A_{xxxnm}(\text{time 1}) - A_{xxxnm}(\text{time 2}) \quad \textbf{(Equation 3)}$$

3.11.1. Esterase activity assay

Assay was carried out as described by Wilhelm *et al.* (1999) [28] using a *p*-nitrophenyl caproate (pNPC) as substrate for determination of the esterase activity. In short, the substrate solution was prepared by vigorous stirring of 1 ml of pNPC stock solution (0.5% (v/v) in absolute ethanol) with 20 ml of potassium phosphate buffer (72 mM $K_{2\,h}PO_4$, 28 mM KH_2PO_4, 10 mM $MgSO_4$, pH = 7.2) followed by incubation for 5 minutes at 30°C. The reactions were started by adding the enzyme sample to the substrate solution at 30°C and measuring the absorbance at 410 nm spectrophotometrically. The enzyme activity was calculated using the molar extinction coefficient of p-nitrophenolat: ε = 10400 $M^{-1}cm^{-1}$.

3.11.2. Lipase activity assay

The lipase activity was measured using the *p*-nitrophenyl palmitate (*p*NPP) as a substrate according to a modified Winkler and Stuckmann method (1979) [169]. The substrate solution was prepared by mixing 5 ml of *p*NPP stock solution (8 mM in propan-2-ol) with 50 ml of the assay buffer (50 mM Na_2HPO_4, 50 mM KH_2PO_4, 5 mM sodium deoxycholate and 0.1% (w/v) gummi arabicum). The reaction was started mixing the substrate solution with the enzyme sample at 37°C and measuring the absorbance at 410 nm spectrophotometrically.

3.11.3. Lysophospholipase A1 activity assay

Lysophospholipase A1 (LysoPLA1) activity was measured using the 1-monopalmitoyl phosphatidylcholine as a substrate [123, 150]. The first step of assay involves the hydrolysis of the lipid substrate upon the activity of PLB and LysoPLA. The second step implies the quantification of liberated free fatty acids by means of the NEFA-HR2 kit. In this step are involved three processes: synthesis of acyl-CoA, oxidation of acyl-CoA and reduction of hydrogen peroxide driven by acyl-CoA synthetase, acyl-CoA oxidase and peroxidise, respectively (see instruction manual). The final product quinoneimine is absorbing at wave length of 550 nm.

The reactions were started combining the 25 µl of lipid substrate (6 mM) in 3 mM NaN_3, 0.5% (v/v) Triton X-100, and 20 mM Tris-HCl (pH 7.2) with 25 µl of enzyme sample (total cell extract, supernatant or pure protein). Prior to incubation, the lipid substrates were vortexed for 15 min at 37°C and then exposed to ultrasonication three times for 20 seconds. The incubations were performed at 37°C with agitation at 250 rpm in Eppendorf termomixer. The assay was modified for the use of 96-well micro-plates, i.e. 2 µl to 10 µl of the reaction mixture was mixed with the 50 µl of reagent A following by the incubation for the 10 minutes at 37°C. After the incubation 100 µl of reagent B was added and the absorbance at 550 nm was measured [123].

3.11.4. Phospholipase A1 and A2 activity assays

The phospholipase A2 (PLA2) activity was measured with the sPLA2 Assay Kit according the producer instruction. The assay is colorimetric based on the hydrolysis of 1,2-dithio diheptanoyl

phosphatidylcholine. Phospholipase A1 activity was measured with the same kit as PLA2 activity was measured but as a substrate was used 1-thiopalmitoyl 2-palmitoyl phosphatidylcholine. Upon enzymatic hydrolysis of the thioester bond of substrate the reaction between free thiol group and the DTNB take a place yielding the chromophore 5-thio-2-nitrobenzoat (TNA). The increase in concentration of TNA directly is proportional to the decrease of substrate concentration.

Assay was performed at 30°C by mixing the 10 μl DTNB (10 mM in 0.4 M Tris-HCl, pH = 8), 5 μl of assay buffer (25 mM Tris-HCl, pH = 7.5, 10 mM CaCl, 100 mM KCl, 0.3 mM Triton X-100), 10 μl of enzyme sample with the 200 μl of substrate solution (1.66 mM substrate). Enzymatic reaction was monitored at wave length of 414 nm. The enzyme activity was calculated using the molar extinction coefficient of 5-thio-2-nitrobenzoat: ε = 13600 $M^{-1}cm^{-1}$.

3.11.5. Thioesterase activity assay

The activity towards acetyl Coenzyme A and palmitoyl Coenzyme A was determined spectrophotometrically according to Bonner and Bloch (1972) [170]. In short substrate solutions contains 20 μM substrate, 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 0.2 M KCl in 50 mM Tris-HCl buffer (pH = 7.5) was mixed with the enzyme samples and the activity was followed by measuring the absorbance at 412 nm. Coenzyme A liberated upon the thioesterase activity reacts with the DTNB yielding the coloured 5-thio-2-nitrobenzoat. The enzyme activity was calculated using the molar extinction coefficient of 5-thio-2-nitrobenzoat: ε = 13600 $M^{-1}cm^{-1}$.

3.11.6. Protease activity assay

The "Protease Colorimetric Detection Kit" was used to detect the protease activity according the supplier instructions (Chapter 2.7). This kit is aimed for detection of serine, cysteine, metallo and aspartic proteases. The assay principle is to hydrolyse a caseine (substrate) by the proteases in order to generate the peptides. Afterwards the peptides which contain tyrosine and tryptophan residues were extracted by trichloracetic acid and subjected to the reaction with the Folin-Ciocalteu (FC) reagent. During this reaction Tyr and Trp residues reacts with FC reagent resulting with the appearing of blue colure measured on λ = 660 nm.

The assay was performed in cuvettes incubating 130 μl of substrate solution with 25 μl of enzyme sample for 10 min at 37°C. After the incubation 130 μl of TCA solution was added to the samples and they were centrifuged for 5 min at 10000*g*. Furthermore, 250 μl of supernatant was incubated with the 625 μl of the Na_2CO_3 and 125 μl of FC reagent for 30 min at 37°C. After the incubation the absorbance at λ = 660 nm was measured.

3.11.7. β-lactamase activity assay

The β-lactamase activity was measured with chromogenic substrate from the group of cephalosporins, CENTA , which was successfully applied for determination of activity of numerous β-lactamases [171], here a modified method was used. In short, substrate solution was prepared dissolving sodium salt of CENTA in potassium phosphate buffer (100 mM, pH = 7.2) yielding a 150 μM concentration of CENTA. The enzymatic reactions were started combining 10 μl of sample and 150 μl of substrate in 96-well plate and absorbance at wave length of 405nm was continuously recorded at 30°C. In the calculations was used extinction coefficient (ε = 6400 M^{-1} cm^{-1}) of hydrolytic product released upon the hydrolysis of CENTA.

3.11.8. Zymographic method for determination of esterase activity

Zymography is an electrophoretic technique that allows detection of enzyme activity. The SDS PAGE electrophoresis under denaturating conditions was performed like was described previously (3.10). Following the protein migration SDS PAGE gel was incubated for 1 h with enzyme-renaturation buffer (100 mM Tris-HCl pH = 8, 25% (v/v) propan-2-ol) which allows the enzymes present in the gel to become active. After the renaturation of enzymes gel was incubated for 2-10 min in 5 mM substrate solution, 4-Methylumbelliferyl butyrate (MUB) dissolved in enzyme renaturation buffer [172]. The gel was photographed under the long wave UV light to record the fluorescence representing the esterase activity.

3.12. Inhibition of proteins

The inhibition of hydrolases was tested using the PMSF (typical serine protease inhibitor), THL (pancreatic lipase inhibitor) and the EDTA (metaloenzyme inhibitor). The 100-fold stock solutions of inhibitors were prepared dissolving the PMSF in DMSO, THL in propan-2-ol and EDTA in water. The enzyme inhibition was performed incubating the enzyme aliquots with the different concentrations of inhibitor on ice. Afterwards the residual activities were measured and the percentage of the inhibition was calculated (Equation 4) based on the activity of untreated sample which was taken to have 100% of activity. The untreated sample corresponds to the enzyme incubated with the same amount of solvent in which were dissolved inhibitors.

$$\text{Residual activity (\%)} = \left(\frac{Activity_{Inhibited}}{Activity_{Untreated}} \cdot 100 \right) \qquad \textbf{(Equation 4)}$$

3.13. Temperature stability of proteins

Temperature stability was tested by incubating the enzyme solution for 30 min at different temperatures following with the measurement of enzyme activity. The enzymes aliquots of 50 μl were incubated at different temperatures in the PCR termocycler (Eppendorf). Following incubation aliquots were transferred to the 96-well plate where the activity was measured in kinetic mode (during 15 min with step of 20 sec).

3.14. Fractionation of *P. aeruginosa* cells

3.14.1. Separation of cells and supernatant

In order to analyze total cell fraction the cultures were centrifuged for 1 min at 19000*g*. Harvested cells were resuspended in 100 mM Tris-HCl buffer (pH 8) to the 15 U_{Cell}/ml. The samples subjected to the enzyme activity assay were sonicated twice for 2 minutes on ice.

3.14.2. Separation of membranes, cytoplasm, periplasm and the supernatant[173]

The *P. aeruginosa* cells were harvested by the centrifugation for the 10 min at 3000*g* and 4°C. Supernatant was separate from the cells and the residual cells were removed by the filtration trough the filter with the pore size of 0.2 μm. To release periplasmic proteins cells were resuspendate in Tris-HCl buffer (100 mM, pH = 8) supplement with 10% (w/v) sucrose at final cell density of 10 U_{Cell}. Afterwards the same volume of Tris-HCl buffer (100 mM, pH = 8) supplement with 10% (w/v) sucrose

and 5 mM EDTA was added. To the cell suspension lysosyme was added (150 *U* per 1 U_{Cell}) and cells were incubate for 30 min at room temperature with gently shaking. The periplasmic proteins released in the supernatant were removed from the spheroblasts by centrifugation for 20 min at 10000*g* and 4°C. The speroblasts were fragmented by the means of ultrasound. The inclusion bodies and the cell debris were separate by the centrifugation for 10 min at 3000*g* and 4°C. Further separation of cytoplasmic proteins from the membranes was conducted by centrifugation for 2 h at 47000*g* (SS-34 rotor) or 30 min at 180000*g* (TLA-55 rotor). The pellet contains total cell membrane fraction including the outer and inner membranes [28].

3.14.3. *In vitro* separation of inner and outer membranes [173, 174]

Equilibrium sedimentation in the gradient of a sucrose solution was used for separation of inner from outer membranes. The method is based on different density of outer (higher density) and inner (lower density) membranes. During the centrifugation of total cell membranes placed on the top of the gradient each membrane fraction proceed until it reaches an environment of comparable density. The forming of disks like areas enriched in inner and outer membranes could be visible by eyes due to the different coloration of inner (brownish) and outer (white) membranes. The advantage of that method over the detergent solubilisation methods is isolation of native membrane proteins.

The total cell membranes were isolated from 200 U_{cell} by centrifugation as described in 3.13.3 with the slight changes. The cells were resuspendated in 10 mM Tris-HCl (pH = 7.4) buffer supplement with the 10 μg/ml RNAse, 10 μg/ml DNAse and 1 mM PMSF and subjected to the cell disruption by means of French press followed by the ultracentrifugation as described in 3.13.2. Membranes were resuspendate in 0.5 ml Tris-HCL buffer (10 mM, pH = 7.4) supplement with 5 mM EDTA, 20% (w/v) sucrose and 20 tablet/l of one fold complete EDTA free protease inhibitor cocktail (Roche). The discontinues sucrose gradient was build up in stepped manner using the 1.8 ml of 20%, 30%, 35%, 40%, 45%, 50% and 55% (w/v) sucrose in the same buffer as was used for membranes solubilisation. The total membrane fraction was loaded to the top of the gradient tube and the system was temperate at 4°C. The centrifugation was carried out in vacuum ultracentrifuge for 72 h at 4°C and 274000*g* (SW41 rotor). After the separation 700 μl fractions were collected and subjected to the SDS PAGE and immunological analysis.

3.15. Separation of integral and peripheral membrane proteins

To differentiate integral from peripheral membrane proteins, cell membranes were incubate for 30 min at room temperature with three different reagents: 10 mM Na_2CO_3 (pH = 11), 4M Urea (in 20 mM MES buffer pH = 6.5) and 2% (w/v) Triton X-100 (in 20 mM MES buffer pH = 6.5). After the incubation the samples were centrifuged for 30 min at 180000*g* to separate insoluble proteins (integral) from soluble proteins (peripheral). So obtained membrane fractions may be analysed by SDS PAGE or activity assays.

3.16. Protein purification by metal-affinity chromatography

Proteins tagged with the carboxy terminal His6-tag were purified by means of nickel-nitrilotriacetic acid metal-affinity chromatography (Ni-NTA MAC) [172]. All manipulations with native protein samples were carried out at 4°C. In this work self made gravity flow columns loaded with 1-2 ml of Ni-NTA agarose superflow resins (QIAGEN) and the XK 16 chromatographic column (Pharmacia)

loaded with 7 ml resins were used. The gravity flow columns were pre-equilibrated with 5 columns volumes following with the loading of sample, equilibration with 5 columns volume, washing with 5 column volumes finally the target protein was eluted with 2.5 columns volumes. In the equilibration, washing and elution steps were used equilibration, washing and elution buffer respectively (chapter 2.5). The purification with the XK 16 chromatographic column was performed on the system with the peristaltic pump (P1 Pharmacia), optical unit (single path monitor UV-1, Pharmacia) control unit (UV-1, Pharmacia) and the printer (LKB REC101 Pharmacia) or using an Äkta explorer 100 system (Pharmacia Biotech). The stepwise purification was performed with the equilibration, washing and the elution buffers performing each step as long as no more proteins were eluted from the column.

The purification of proteins isolated from inclusion bodies were performed under the denaturating conditions at room temperature. Prior the purification the inclusion bodies were treated for 1 h with the equilibration buffer containing 8 M urea. The procedure was the same like for purification of native proteins with the difference in the presence of 8 M urea in each buffer [175].

3.17. *In vitro* renaturation of proteins

The heterologous overexpression of protein sometimes leads to the formation of the insoluble protein aggregates called inclusion bodies. To recover the protein from inclusion bodies in its native conformation is challenging and still empirical process. As the folding of the proteins is affected by number of physiochemical parameters the testing of different conditions needed to refold a protein is on time common method to refold a protein. These parameters include: Ionic strength, pH, temperature, oxidation state and protein concentration as well as the presence of hydrophobic, polar, chaotropic agents and other proteins.

The used method involves denaturation of the inclusion bodies with 8 M urea during the 3 h at the room temperature. Afterwards the aliquots of denaturated inclusion bodies were diluted with 20 fold excess of renaturation buffers (Table 10) and incubate over night at 4°C. The success of renaturation was tested by means of activity assay.

Table 10: Buffers used for *in vitro* renaturation.

Number	Tris-HCl pH = 8.5 / mM	NaCl / mM	KCl / mM	Triton X-100 / (v/v)	Sucrose / M	Arginine / M
1	50	9.6	0.4	0.05%	0.4	0.5
2	50	9.6	0.4	0.05%	0.4	-
3	50	9.6	0.4	0.05%	-	-
4	50	240	10	0.05%	0.4	0.5
5	50	240	10	0.05%	0.4	-
6	50	240	10	0.05%	-	-

3.18. Concentration of proteins.

3.18.1. Protein concentration under the native conditions

The protein samples used in activity assays were concentrated by means of ultrafiltration using the Vivaspine centricons with the defined pore size of 5 kDa or 10 kDa according the supplier instructions. The same method was used to exchange a buffer in which proteins were dissolved.

3.18.2. Precipitation of proteins.

Proteins were precipitated using the modified Peterson method (1983) [176]. In short, protein samples were mixed with the 50% (w/v) trichloroacetic acid in acetone giving the 10% (w/v) end concentration of TCA. After the incubation on ice for at least 1 h samples were centrifugat for 45 min at 19000*g*. The precipitate was washed for two times with 1 ml 80% (v/v) acetone and dried at room temperature or in the vacuum centrifuge. The proteins were dissolved in desired volume of 100 mM Tris-HCl buffer (pH = 8) by incubation for 10 min at 60°C.

3.19. Determination of protein concentration.

The protein concentration was determinate using the Bradford assay [177]. The protein sample (100 μl) was mixed with the 900 μl of Bradford reagent and incubated for 5 min at room temperature following with measurement of absorbance at 595 nm. The standard curve was made using the bovine serum albumin.

3.20. Identification of proteins by matrix assisted laser dissociation-ionization mass spectrometry.

Using the sensitive method, as it is matrix assisted laser dissociation-ionization mass spectrometry (MALDI MS), it is feasible identification of single protein after separation of proteins on SDS PAGE gel and visualization by Coomassie staining. The protein identification implies five steps:

a) removing the Coomassie dye from proteins

b) digestion of protein

c) extraction of peptides

d) detection of peptides by MALDI mass spectrometry.

e) data processing and identification of peptides, proteins.

The small amount of protein was collected together with the gel cutting the band of desire out of the SDS PAGE gel. The gel slice was than incubated with 350 μl of acetonitrile (30% v/v) containing 0.1 M ammonium dihidroxycarbonate during 20 min at room temperature in agitation mode. The liquid was removed and previous step was repeat until the gel was colourless. The transparent piece of gel was dried under the vacuum at the room temperature for the 20 minutes. The dried gel was incubated with 3 μl of trypsine solution (19.6 ng/μl in 3 mM Tris-HCl, pH = 8.8) for 30 min at room temperature. After that 3 μl of Tris-HCl buffer (3 mM, pH = 8.8) was added to the gel and it was incubated overnight at room temperature in order to digest the protein to the smaller peptides. The peptides were extracted from the gel by incubation of gel with 2 μl of water (milipore grade) for 15 minutes at room temperature following by incubation of gel with 5 μl of acetonitrile (30% v/v)

contains TFA (1% v/v) for 15 minutes at room temperature in ultra-sonification water bath. The prepared samples may be directly applied to MS MALDI analysis or may be stored at -20°C. The 1 µl of peptide solution was applied on the PAC target spot. After 3 minutes 7 µl of ammonium phosphate (10 mM aqueous solution) with TFA (0.2% v/v) was applied to the protein spot and liquid was immediately removed by soaking it with the paper tissue. The completely dried protein samples were analyzed by MS MALDI method.

The MS MALDI analysis, data processing and search of protein database were performed at the Institute for Biotechnology, Research Center, Juelich.

3.21. Immunological detection of proteins

3.21.1. Western blot

The proteins were transferred from SDS PAGE gel to the PVDF membranes by means of western blot [178] using the Mini Trans Blot Electrophoretic Transfer Cell (BioRad). Prior the transfer, membranes were shortly incubated in the methanol following the washing in the water and equilibration in the dunn carbonate buffer. The SDS PAGE gel was also equilibrated for 5 min in dunn carbonate buffer. The protein transfer was carried out in two steps procedure, first 15 min at 150 mA followed by 20 min at 300 mA (constant electric current) using the dunn carbonat buffer and appropriate cooling system.

3.21.2. Immonodetection of protein on PVDF membranes

After the protein transfer the membrane was socked for 1 h in the TBST or PBST buffer contains skim milk in order to block protein-free positions of the membrane. After the blocking membrane was washed with TBST or PBST buffer and incubated with the antibodies. The Anti-His(C-term)-HRP antibodies (Invitrogen) and Anti·His antibodies (Qiagen) and HRP conjugated Goat Anti Mouse IgG/HRP (Jackson Immunoresearch) were handled according the manufacturer instructions. The anti-rabbit polyclonal antiserums were used to detect different proteins (Table 11). All steps were carried out at 30°C. The procedure involves blocking of the membrane with the TBST (2% (v/v) Tween 20) supplement with 3% (w/v) skim milk for at least 1 h. Blocking of the membrane by incubating it for 1 h with the TBST buffer followed by the incubation for 1 h with the antibodies serum diluted with TBST. Afterwards the membrane was washed twice for 30 min with the TBST and incubated for 1 h with the secondary Anti-Rabbit IgG antibodies coupled to HRP (Promega). The last washing step involves two times incubation for 30 min and two times incubation for 5 min with the TBST. In this step non-specific bounded secondary anti-bodies should be removed what result with the lower level of the background intensity. Therefore, sometimes the additional washing with the TBST for 5 min was applied (2-3 times).

Table 11: Polyclonal antibody serums used in this PhD work

Protein	Anti-bodies source	Dilution	Reference
DsbA	Rabbit	1/50000	[179]
XcpQ	Rabbit	1/5000	[180]
XcpZ	Rabbit	1/2000	[181]
ToxA	Rabbit	1/5000	[182]

3.22. *Drosophila melanogaster* virulence model

Virulence of *P. aeruginosa* strains were tested in common virulence model using the *D. melanogaster* as a host. The needle pricking assay was performed as described in Apidianakis and Rahme (2009) [183]. The assay and pathogenesis assessment was performed at the Harvard Medical School, Department of Surgery, Boston (USA) by Rien Hoge (Institute for molecular enzyme technology, Heinrich-Heine University, Duesseldorf, Germany).

3.23. Internet databases and softwares

Pseudomonas Genome Database [19]:
- DNA and protein sequences
- Gene and protein annotation
- BLAST sequence homology search using BLINK tool

Pfam database (releas 24.0) [184]:
- Prediction of protein domains and their organisation
- Visualisation of Hidden Markov Models (HMMs)

Protein data bank [185]:
- Protein crystal structures

UniProt Knowledgebase at ExPASy Proteomics Server:
- Annotation of protein functions and active sites
- Multiple sequence alignments
- MyDomains domains visualisation tool [186].

EMBOSS Pairwise Alignment Algorithm
- Global and Local alignment of protein (default parameters)

Phyre webserwer [187]:
- Protein structure prediction from sequence

SignalP server [188]:
- Signal peptides prediction in proteins

PSORTb (version 2.0) [189]:
- Prediction of subcellular localisation of proteins

Mobyle portal [190]:
- Prediction of transmembrane regions and their orientations by Sigcleave program

MEMSAT3 [191]:
- Prediction of transmembrane regions and their orientations by Sigcleave program

TMpred [192]
- Prediction of transmembrane regions and their orientations

Toppred [193]:
- Prediction of transmembrane regions and their orientations

SoftMAX Pro (version 3.1.2) and Excel (Microsoft office)
- Analysis of kinetic data obtained with 96-well microplate reader SpectraMax 250

SwisPDB viewer (version 4.0.1)[194]
- Analyses and visualisation of protein structures

Advance image data analyser (AIDA) 2D software (version 4.18.028)
- The densitometric quantification of Western blot signals

4. Results

4.1. *P. aeruginosa* PA01 expresses phospholipase A2 and lysophospholipases A

The major aim of this PhD work was to examine whether *P. aeruginosa* PA01 expresses phospholipases A (PLAs) and lysophospholipases (LysoPLAs) and furthermore, to identify the genes that encode these enzymes.

To achieve our aim, we first examined wild-type *P. aeruginosa* PA01 for expression of enzymes exhibiting PLA2 and LysoPLA activity. To obtain a better overview of cellular localization of putative PLA and LysoPLA, each cell compartment was examined separately. *P. aeruginosa* PA01 cells grown in LB under standard conditions (Chapter 3.1.2) until late logarithmic phase were fractionated (Chapter 3.14.2), yielding cytoplasmic, periplasmic, membrane and extracellular protein fractions. The purity of the fractions was gauged by immunodetection of periplasmic protein (DsbA) [179], outer membrane protein (XcpQ) [180] and extracellular protein (ToxA) [195] in each cell fraction (Figure 10B). Presence of signal corresponding to DsbA, XcpQ and ToxA exclusively in periplasmic, membrane and extracellular fractions, respectively, indicated a satisfactory purity of each fraction. The PLA2 and LysoPLA activity of each fraction was measured (Figure 10A). To compare the activities of fractions between themselves, equal amounts of each fraction (equivalent to a defined amount of cells) were used in the assay. All cell fractions were prepared in the same buffer (supernatant was diluted with a corresponding buffer); therefore, non-specific hydrolysis of substrate upon incubation with buffer was subtracted from each measurement. The PLA2 and LysoPLA activity profiles (Figure 10A) indicate that activity was mainly detected in periplasmic (57% and 48%) and membrane (38% and 35%) fractions. The LysoPLA activity of cytoplasm (9.8%) and supernatant (7.7%) were just above the detection limit of the LysoPLA assay (ca. 5%). The PLA2 activity in cytoplasmic (3.1%) and extracellular (2.7%) fractions was minor with respect to the detection limit of the PLA2 assay, which was 1%. Because of the marginal PLA and LysoPLA activities measured in the cytoplasmic fraction, it is likely that cytoplasmic proteins do not contribute to the PLA and LysoPLA activities measured in the other three fractions. Therefore, the lack of an antibody to identify the cytoplasmic fraction by western blot does not introduce uncertainty into the interpretation of the experiment.

These data are the first evidence that *P. aeruginosa* PA01 expresses enzymes with PLA2 and LysoPLA activity. Moreover, the data presented clearly indicate the major portion of PLA2 and LysoPLA activities are associated with the membrane and periplasmic fractions of *P. aeruginosa* PA01. Because of the low PLA2 and LysoPLA activities measured in the supernatant, it is not obvious whether *P. aeruginosa* PA01 secretes PLA2 or LysoPLA under the conditions chosen here. As it is known that some PLA2 may also posses LysoPLA activity [196], it is challenging to demonstrate the specificity of isolated membrane bound and periplasmic enzymes to PLA2 and LysoPLA substrates.

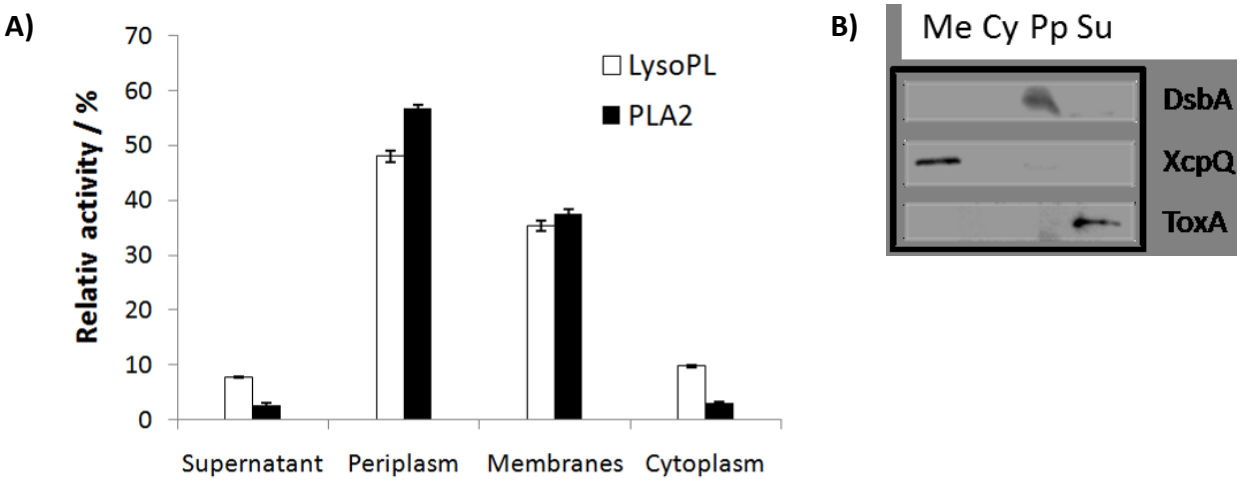


Figure 10: PLA2 and LysoPLA activities of *P. aeruginosa* PA01 cell compartments. **A)** Lysophospholipase A and phospholipase A2 activities were measured with equivalents of 0.025 U_{Cell} and 0.015 U_{Cell}, respectively. The relative activities were calculated by dividing the absolute activity of a particular fraction by the sum of the absolute activities of all four fractions. **B)** Western blot of cell fractions using the antibodies against periplasmic protein (DsbA, Mw(theoretical) = 21.1 kDa), membrane protein (XcpQ, Mw(theoretical) = 66.4 kDa) and extracellular protein (ToxA, Mw(theoretical) = 66.7 kDa). The SDS PAGE gel contained 0.15 U_{Cell} of total membrane (Me), cytoplasm (Cy) and periplasm (Pp) and 0.5 U_{Cell} of supernatant (Su).

4.1.1. Expression of PLA2 activity during *P. aeruginosa* PA01 growth

To assess whether the expression of PLA2 activity in *P. aeruginosa* PA01 is a function of cell density, the PLA2 activity of the bacterial culture was measured at three different growth stages (early logarithmic, middle logarithmic and late stationary phases). It is now well understood that the cell-to-cell signalling system ("Quorum sensing") of *P. aeruginosa* allows bacteria to produce proteins and metabolites in cell density-dependent manner [8]. A variety of cell-associated (lipopolysaccharides, alginate, flagellum, pilus) and extracellular (phospholipase C, proteases, exotoxin A and S, rhamnolipids, pyocyanin) virulence factors are controlled by this regulatory signalling system [8]. The hypothesis that PLA could be novel virulence factor of *P. aeruginosa* PA01 raised the question of whether PLA activity depends on the cell population density.

P. aeruginosa PA01 culture was grown in rich LB medium (Chapter 3.1.2), and samples were collected and analysed 2 h, 4 h and 30h (early logarithmic, middle logarithmic and late stationary phases, respectively) after inoculation. Previously, we showed that PLA2 activity in *P. aeruginosa* PA01 is predominantly cell-associated (Figure 10A). Therefore, further measurements of PLA2 activity were performed with total cell lysates (Figure 11). The data show that *P. aeruginosa* PA01 exhibits PLA2 activity at all three growth stages. The activity in the early logarithmic culture was 35% of that of the stationary culture, and the activity in the middle logarithmic culture was 65% of that of stationary culture. These data demonstrate that *P. aeruginosa* PA01 exhibits PLA2 activity at all growth stages. Furthermore, PLA2 activity increases until the late stationary phase up to three fold compared to the early logarithmic phase.

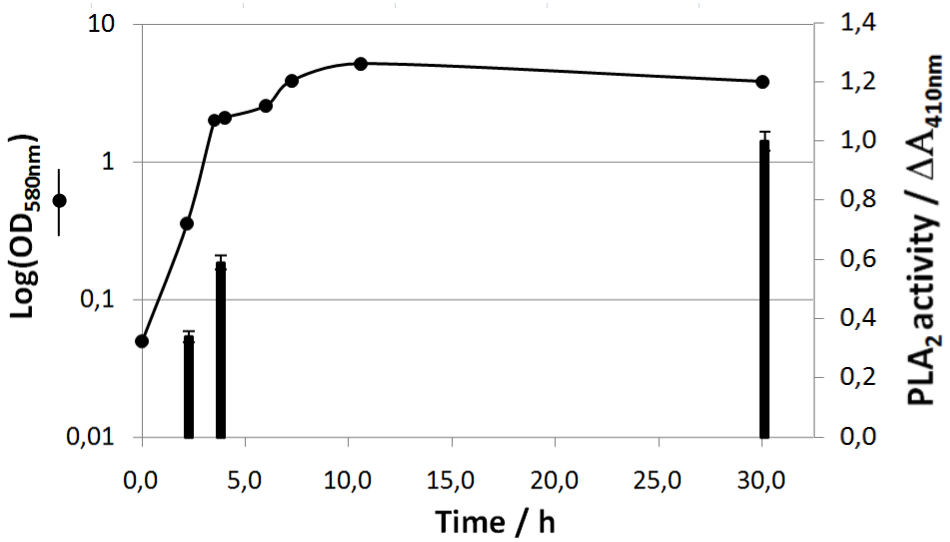


Figure 11: Expression of PLA2 activity during growth of *P. aeruginosa* PA01. PLA2 activity was measured with 0.05 U_{cell} of total cell lysates of cultures at OD_{580nm} = 0.36, 2.58 and 3.86. The results are the mean of two measurements performed in triplicates.

4.1.2. Effect of growth medium on PLA2 activity in *P. aeruginosa* PA01

To test whether PLA2 activity in *P. aeruginosa* PA01 is affected by growth media, cultures were grown in rich medium (LB), chemically defined minimal medium (MME) and complex phosphate limitation medium (PPGAS). The cultures were grown overnight at 37˚C (Chapter 3.1.2) to ensure stationary cultures. Cells were disrupted by sonication and PLA2 activity was measured in the total cell lysate (Figure 12). The PLA2 activity of the culture grown in rich medium was more than double that of cultures grown in nutrient limited medium (MME and PPGAS). Furthermore, the PLA2 activity of culture grown in minimal media (MME) was decreased compared to the culture grown in richer PPGAS medium. These results indicate that limitation of nutrients negatively affects PLA2 activity in *P. aeruginosa* PA01. One possible explanation could be that PLA2 is required during lipid metabolism or nutrient utilisation, and therefore, expression of PLA2 in LB medium is higher than in minimal medium.

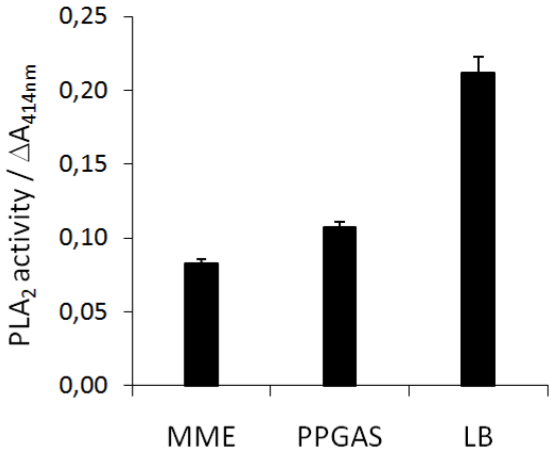


Figure 12: PLA2 activity of *P. aeruginosa* PA01 grown in different media. 0.01 U_{cell} of total cells lysates of stationary phase cultures grown in MME, PPGAS and LB were subjected to the PLA2 assay. The A_{414nm} was continuously recorded during 10 min of enzymatic reaction. The results are the means of two measurements performed in triplicate.

4.1.3. Comparison of the PLA2 activity of wild type and *estA* negative mutant *P. aeruginosa*

We demonstrated for first time that wild type *P. aeruginosa* PA01 exhibits PLA2 activity. Unknown enzymes must contribute to this activity because no protein with PLA2 activity has previously been described in *P. aeruginosa* PA01. However, it has been indicated that membrane bound esterase, EstA [28], exhibits phospholipase B activity. Phospholipases B are enzymes that display PLA1 and PLA2 hydrolytic activities. In the present study, we quantitatively measured the PLA2 activity of pure EstA (Figure 13B). Previously, it was published that expression of full-length EstA in *E. coli* yields inclusion bodies from which EstA can be recovered in enzymatic active form by using refolding buffer (Tris-HCl 20 mM, pH 8.0 suplemented with 0.5% (w/v) N-dodecyl N,N-dimethyl-1-ammonio-3-propanesulphonate (SB-12)) [197]. Because the EstA purification procedure includes reactivation of inactive EstA, an esterase assay (pNPC assay) was performed to control for proper functionality of EstA. The esterase activity measurements confirmed that reactivated EstA is catalytically active (data not shown). In the subsequent measurements of PLA2 activity, a continuous increase of A_{414nm} was observed as a result of hydrolysis of the substrate by EstA (Figure 13A). These data demonstrated that EstA indeed possesses PLA2 activity against the substrate chosen here.

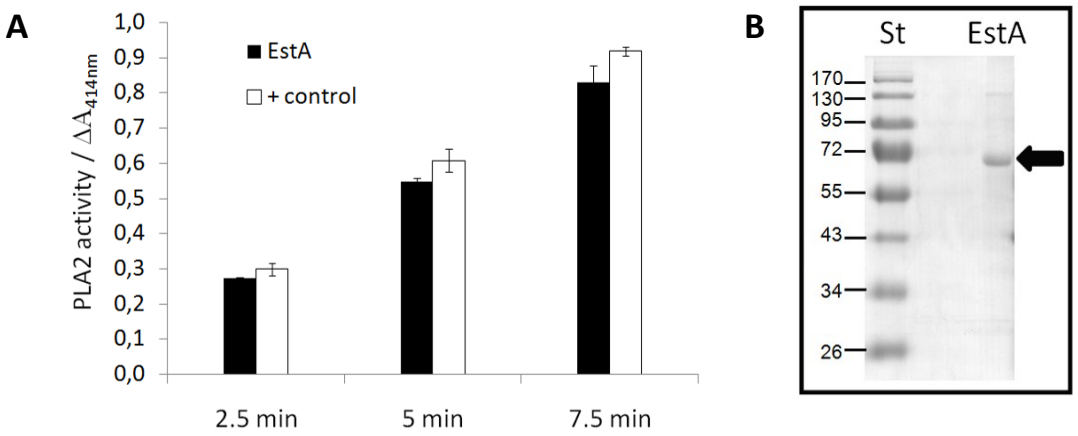


Figure 13: PLA2 activity of EstA. **A)** A PLA2 activity assay was performed with 68 ng of refolded EstA. In the positive control reaction, 10 ng of *Bee venom* PLA2 (supplied with the assay kit) was used. The negative control (denatured EstA) did not show any activity. The results are the mean of three measurements. **B)** Coomassie stained SDS PAGE gel (14%) shows the purity of EstA used in PLA2 activity measurements. The black arrow indicates EstA.

Because EstA was shown to exhibit PLA2 activity, the contribution of EstA to the total PLA2 activity of *P. aeruginosa* PA01 was measured. Therefore, the PLA2 activities of total cell lysates of *P. aeruginosa* PA01 (wild type) and PASCHI (*estA* negative mutant) [1] grown to stationary phase were compared. Consequently, partial fractionation of both strains was performed yielding supernatant, a total membrane fraction and a soluble fraction, representing periplasmic and cytoplasmic proteins. The fractions were subjected to the PLA2 activity assay (Figure 14). The results demonstrated cell-bound PLA2 activity in both strains. In the supernatant, PLA2 activity was not detectable. The PLA2 activity in total cell lysate of the PASCHI strain was 13% lower than wild type. Roughly the same difference in PLA2 activity was observed by comparing the membrane fractions of wild type and PASCHI (12.7% lower activity in PASCHI). Furthermore, the measured PLA2 activities present in soluble fractions of wild type and PASCHI were nearly the same. We can conclude that lack of EstA protein in the PASCHI

strain influences the decrease in PLA2 activity in total cells and the membrane fraction but does not affect the PLA2 activity of the soluble fraction. The results presented here led to the conclusion that in addition to EstA, *P. aeruginosa* PA01 expresses additional membrane bound and soluble proteins with PLA2 activity. Therefore, it is tempting to identify novel enzymes that contribute to the PLA2 activity of *P. aeruginosa* PA01.

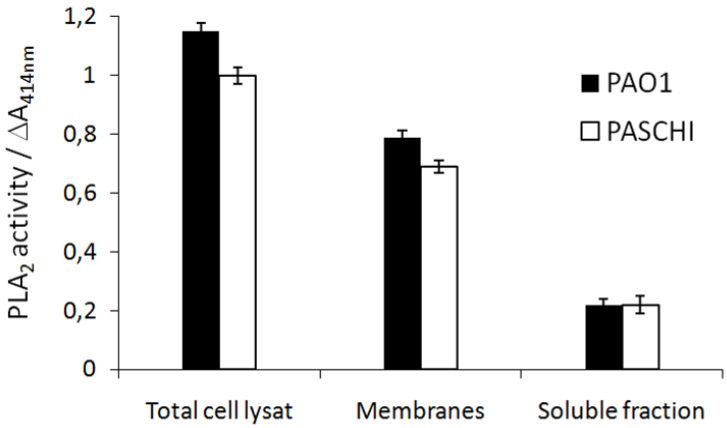


Figure 14: PLA2 activity of *P. aeruginosa* PA01 (wild type) and PASCHI (*estA* negative mutant) strains. The equivalent of 0.05 U_{Cell} of total cell lysates, membranes and soluble fractions were used in measurements (200 µl of substrate). The increase in A_{414nm} was continuously recorded during a period of 750 seconds. The results are the mean of three measurements each performed in triplicate.

Summary

4.1 *P. aeruginosa* PA01 expresses phospholipase A2 and lysophospholipases A

We have for first time demonstrated PLA2 and LysoPLA activities expressed in *P. aeruginosa* PA01. These activities are predominantly periplasmic and membrane associated, whereas in the cytoplasm and extracellular room both activities are insignificantly low. Furthermore, we have observed that *P. aeruginosa* PA01 expresses cell associated PLA2 activity in early-logarithmic, middle-logarithmic and stationary growth phase. However, the PLA2 activity gradually increase during the growth, thereby the stationary cells expresses three fold more PLA2 activity than early-logarithmic phase. We have tested influence of nutrient limitation to the PLA2 activity expression using minimal medium (MME), complex phosphate limitation medium (PPGAS) and rich medium (LB). The results reveal decrease of PLA2 activity as a function of nutrient limitation. Thus, MME and PPGAS cultures express only 38% and 50% of PLA2 activity compared to LB culture respectively.

In the light of previous results on outer membrane esterase (EstA) from *P. aeruginosa* PA01, which indicate its possible PLA2 activity [2], we have quantified PLA2 activity of pure EstA protein. For this purpose EstA was expressed in *E. coli* BL21(DE3) and isolated in form of inclusion bodies (Figure 13B) from which it was renaturated. The success of EstA reactivation was confirmed using its known esterase substrate (pNPC). The following PLA2 activity assay revealed that EstA is indeed able to hydrolyze PLA2 substrate (Figure 13A) what point out this enzyme as a first PLA2 described in *P. aeruginosa* PA01. Because, *in vitro* experiment clearly indicates PLA2

activity of EstA we have tested whether *estA* negative mutant strain [1] expresses less PLA2 activity than parental strain. The results indicate that PLA2 activities of total cell lysate and membrane fraction of *estA* negative strain are for 13% lower comparing to the wild type. The influence of *estA* deletion on PLA2 activity of soluble fraction was not obtained what is expected because EstA is membrane associated.

Taken together, we can conclude that *P. aeruginosa* PA01 expresses, beside previously described EstA, additional unknown cell associated PLA2 enzymes. These enzymes will be subject of the present study.

4.2. Bioinformatics analysis of lipolytic enzymes

4.2.1. Selection of putative phospholipases in the *P. aeruginosa* PA01 genome

The above outlined observation that *P. aeruginosa* PA01 exhibits PLA2 and LysoPLA activity prompted us to employ bioinformatics methods to identify putative enzymes that might be responsible for this activity. Therefore, we systematically explored the *P. aeruginosa* PA01 genome with the purpose of finding potential phospholipases A and lysophospholipases A. Numerous phospholipases possess esterase and/or lipase activity [107, 198] so esterases and lipases were also included in our search. We decided to take advantage of the annotation of the *P. aeruginosa* genome available in the Pseudomonas Genome Database [11]. This database compiles demonstrated and predicted homologies, functions, structural features, gene ontology and metabolic pathways for each gene. Therefore, we performed a text search of the Pseudomonas Genome Database [19] using the keywords “phospholipase”, “esterase” and “lipase”, criteria that should yield a comprehensive set of enzymes with putative phospholipase function. The search yielded all together 114 genes. Ten of these genes were enzymes previously described in the literature (Table 1). Sixteen of the genes were putative enzymes (Table S1), homologues to the previously described lipolytic enzymes. A majority of enzymes (88) are hypothetical enzymes without homology to previously described enzymes (Table S2).

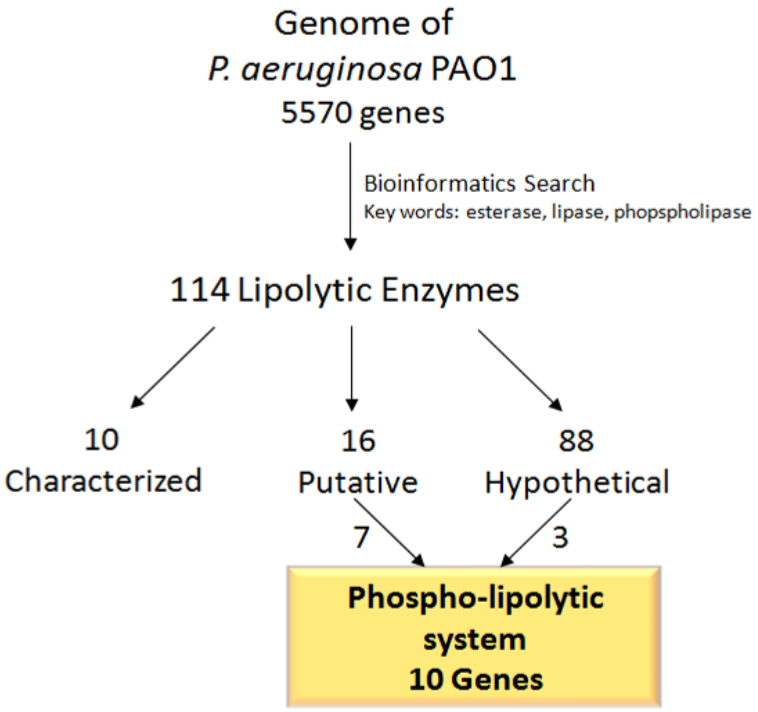


Figure 15: Bioinformatics analysis of the *P. aeruginosa* PA01 genome. Schematic representation of the criteria used to select lipolytic enzymes with potential to be a phospholipase. The characterised enzymes are listed in Table 1 in the Introduction, putative and hypothetical lipolytic enzymes are listed in Table S1 and S2 respectively available in the supplementary data. The most promising candidates, seven putative and three hypothetical proteins, were called in the course of this PhD work phospho-lipolytic system (Table 12).

From the pool of putative and hypothetical enzymes, ten proteins (seven putative and three hypothetical) were selected as the most promising candidates (Table 12). All putative esterases (PA1047, PA2098, PA3628), lipases (PA2949) and phospholipases (PA2155, PA4339) were included in the group analysed here. The exception was a small (113 aa, 12.4 kDa), putative lipase (PA1615) that is unlikely to fill a predicted function. It is an unusually small protein considering that one of the smallest putative hydrolases is YdiI esterase from *E. coli*, which has a size of 136 aa [199, 200]. Furthermore it is predicted to be a GDSL-hydrolase with a conserved $GDS_{60}L$ motif in the middle of its sequence, which is in contrast to the other GDSL-hydrolase in which the GDSL-motif is exclusively found close to the amino-terminal end [4]. Additionally, PA1615 lacks a highly conserved HXXD motif, which contains two members of a catalytic triad (His and Asp) that are crucial for catalysis of GDSL-hydrolases [4]. The putative thioesterase TesA (PA2856) was chosen due to its classification as a member of the GDSL-hydrolase family. This family particularly attracted our attention because of novel sequence features that suggest attractive catalytic properties [4, 3].

From the set of 88 hypothetical lipolytic enzymes, three especially attracted our attention due to their homology to previously described enzymes. First, PA2927 (PlaB) is a homologue of haemolytic phospholipase B from *Legionella pneumophila* [198]. The second one, PA3339 (PlpD), is a putative outer membrane protein that has homology to ExoU, the cytotoxic phospholipase A2 from *P. aeruginosa* PA14 [107, 43]. The third hypothetical enzyme included in our phospho-lipolytic system is PA4921, which belongs to the GDSL-hydrolase family and possesses homology to the secreted phospholipase A from *L. pneumophila* (PlaA) [123].

Table 12: Putative phospholipases of *P. aeruginosa* PA01 that were the subject of study in the course of this PhD work. The name, *tesA* and putative functions of phospho-lipolytic enzymes are adopted from the Pseudomonas Genome Database [19]. All other genes were named based on knowledge collected in this work.

PA code	Gene name	Putative enzymatic functions
PA1047	*plaK*	Esterase
PA2098	*estB*	Esterase, Deacetylase
PA2155	*pldB*	Phospholipase D
PA2856	*tesA*	Acyl-CoA Thioesterase I
PA2927	*plaB*	-
PA2949	*plbF*	Lipase
PA3339	*plpD*	Lipase
PA3628	*estC*	Esterase
PA4339	*pldC*	Phospholipase D
PA4921	*lipF*	-

4.2.2. Sequence homology and prediction of active sites of phospho-lipolytic enzymes

The activity, cellular localization, regulation and modification of proteins must be explained within biological systems to understand these proteins. Although the experimental approach is evidently the most certain one, it is expensive and time consuming to perform on a large scale. However, the huge amounts of data deposited in gene and protein databases offer a good substitute for the analysis of functions for a large number of enzymes. Prior to experimental studies of phospho-lipolytic enzymes, data mining was performed for each sequence of phospho-lipolytic enzyme, and data about homologous proteins were collected. These data were used as a starting point for the design of experimental approaches to study the phospho-lipolytic system. Although most of the described hydrolases are single domain proteins, they could be organised in modular structures containing more than one domain [201]. The modularity of phospho-lipolytic enzymes was tested by performing a sequence search on the Pfam database. The resulting putative protein domains were defined for each member. Furthermore, a BLAST homology search was performed on the Pseudomonas Genome Database site using the BLINK tool [19]. By screening protein sequences against PDB and SWISPROT databases, two subsets of homologues were defined. Putative active sites and other functional elements were mapped from multiple sequence alignments with a) homologues in which the active sites were described on the level of tertiary/quarterly structure (PDB homologues) and b) homologues without known 3D structures (SwissProt homologues) but with active sites annotated by mutagenesis methods. The demonstrated enzymatic functions of the homologues derived from primary literature were used for the prediction of enzymatic function of phospho-lipolytic enzymes. By this approach, enzymes were assigned to known enzyme families, and the first indications on their possible cellular role were made available. Furthermore, the prediction of catalytic residues could help in the generation of inactive enzyme variants or inhibition experiments. On next pages will be given results for each phospho-lipolytic enzyme separately.

4.2.2.1. Sequence homology of PlaK

A BLAST search performed with the sequence of PlaK showed its homology to esterase and β-lactamase families. Analysis of the primary structure revealed three possible active site motifs: a typical lipase/esterase motif ($GXS_{352}XG$) and two serine β-lactamase motifs ($S_{78}XXK$, $S_{259}XXK$). The GXSXG motif could be a false positive because its localisation close to the carboxy terminal end is not consistent with amino terminal localisation of GXSXG motif in the true lipase family [47]. The sequence alignment of PlaK with the members of the true lipase family (data not shown) revealed no homology in the GXSXG motif, in the lipase catalytic site residues Asp and His or in the Asp residue involved in binding of Ca^{2+} ion that are present in the lipase family [202]. The Pfam database recognised a β-lactamase domain spanning the sequence between aa 25-388 with a predicted active site formed by Ser78 and Tyr167. The catalytic Ser in β-lactamases has a role as a nucleophile that attacks the β-lactame ring, forming an acyl-ester intermediate. The catalytic Tyr serves as a proton acceptor for the hydrogen atom of Ser, activating it for nucleophilic attack [203].

To examine the reliability of prediction of Ser78 and Tyr167 as the active site residues, we aligned the PlaK sequence with the sequences of homologous β-lactamases of which the active sites are described in the literature (Figure 16). The alignment revealed strong conservation of the $S_{78}XXK$ sequence (Figure 16), as opposed to the $S_{258}XXK$ sequence, which is not conserved. Furthermore, the second catalytic residue, Tyr167, is strongly conserved among all members included in multiple alignments (Figure 16). The third sequence motif characteristic for serine β-lactamases (classes A and C) is a KTG tripeptide located between the SXXK motif and the carboxy terminal end [204]. However, this motif was not identified in the PlaK sequence (Figure 16). The function of the KTG tripeptide is not clear yet, but it is known that it builds a wall opposite the active site cleft [204]. Thereby, it may be involved in determination of substrate specificity. Interestingly, EstB from *B. gladioli,* which exhibits esterase but not a β-lactamase activity, also does not have a conserved KTG motif (Figure 16) [205]. Taking the data obtained from Pfam database and multiple alignments together, we can predict with high probability that Ser78 and Tyr167 are part of the active site of PlaK. Even though the sequences of PlaK and the chosen β-lactamases share a maximum 20.5% identity (31% similarity), conservation of the active site residues clearly indicates a functional relationship of PlaK to β-lactamases. Therefore, we have predicted that PlaK may be a β-lactamase or esterase similar to its homologue, EstB from *B. gladioli* [205]

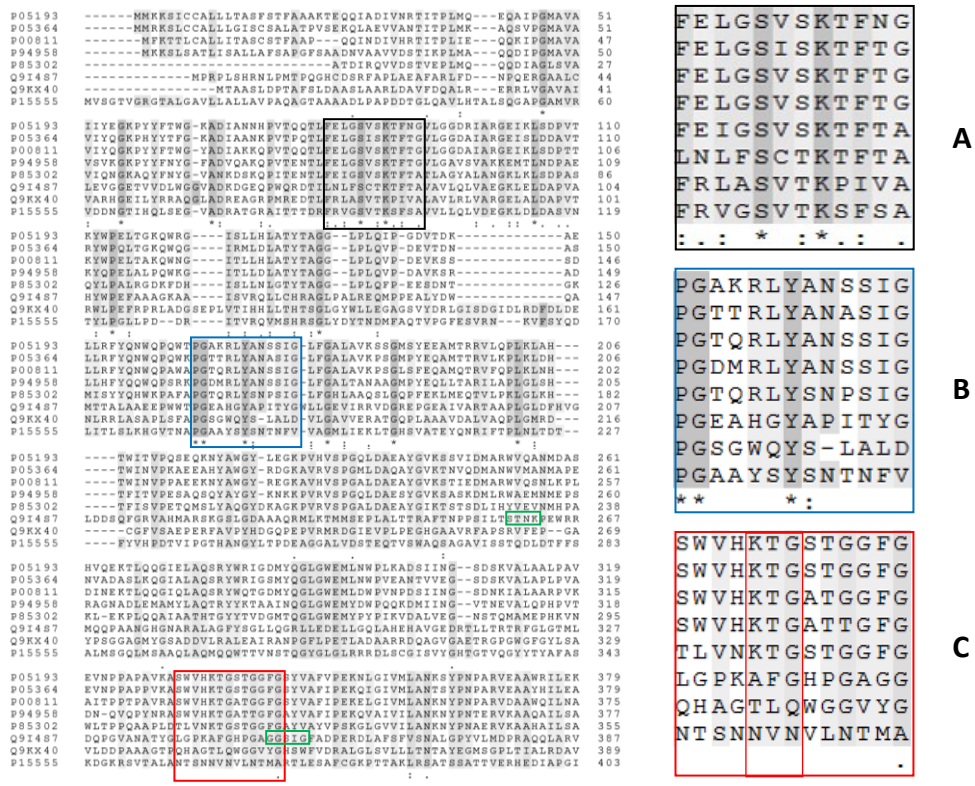


Figure 16: Multiple sequence alignments of (listed from top to bottom) β-lactamase from *Citrobacter freundii* (P05193) [206], β-lactamase from *Enterobacter cloacae* (P05364) [207], β-lactamase from *Escherichia coli* (P00811) [208], β-lactamase from *Morganella morganii* (P94958) [209], β-lactamase from *Pseudomonas fluorescens* (P85302) [210], PlaK (Q9I4S7), Esterase from *Burkholderia gladioli* (Q9KX40) [205] and carboxypeptidase from *Streptomyces* sp. (P15555) [211]. The sequences around the active site Ser and Tyr are presented enlarged in Sections A (black) and B (blue), respectively. Section C (red) represents a sequence around the KTG tripeptide, which is conserved in the β-lactamase family. The sequences representing another two potential active sites of PlaK (S_{259}XXK and GXS_{352}XG) are highlighted in green.

4.2.2.2. Sequence homology of TesA

TesA is the only named member of phospho-lipolytic system according the Pseudomonas Genome Database [19]. The name TesA, meaning thioesterase A, originated from the name of *E. coli* thioesterase (TAP) [3], which shares high sequence homology (62% similarity, 44% identity) to TesA. The TAP enzyme belongs to the GDSL-family [127], which includes more than 5100 members according to the Pfam database [184]. A BLAST search revealed significant sequence homology between TesA (59% similarity, 44% identity) and the arylesterase from *V. mimicus* (VmAest) [212], members of the GDSL-family.

To predict the active site residues of TesA, we performed multiple sequence alignments with enzymes for which the active sites were experimentally annotated. It is known that sequences of GDSL-hydrolases vary considerably outside the active site [4]. Therefore, we have additionally included homologues of TAP and VmAest described in the literature in the multiple sequence alignment despite their low sequence homology to TesA. Although the global sequence alignment reveal low homology, four regions (blocks I, II, III and V) characteristic of GDSL-hydrolases are highly conserved (Figure 17). Each block contained strictly conserved active site residues: catalytic triad Ser

(Block I), catalytic triad Asp and His (Block V), oxyanion hole Gly (Block II) and oxyanion hole Asn (Block III). Using these data, we can predict with high certainty that Ser30, Asp177 and His180 are residues of the catalytic triad of TesA and furthermore, that Gly67 and Asn96 residues form the oxyanion hole. Although the prediction of catalytic residues of GDSL-hydrolases is quit straightforward, the prediction of enzymatic function is rather difficult and unreliable. The reason for this is the heterogeneity of the GDSL-hydrolase family with respect to enzymatic function. For example, hydrolases belonging to this family act on ester bonds in various substrates: thioesters, lipids, esters, arylesters, platelet activating factors, lysophospholipids, phospholipids, polysaccharides and peptides. However, both homologues of TesA are arylesterases [5, 212]; therefore, it is feasible to predict that TesA has arylesterase activity. Furthermore, TAP has been shown to be a multifunctional protein [213, 138], which led us to speculate that TesA might be a multifunctional enzyme (such as thioesterase, lysophospholipase, esterase, protease) as well. Regardless, it must be stressed that reliability of prediction of enzyme function for TesA is not high.

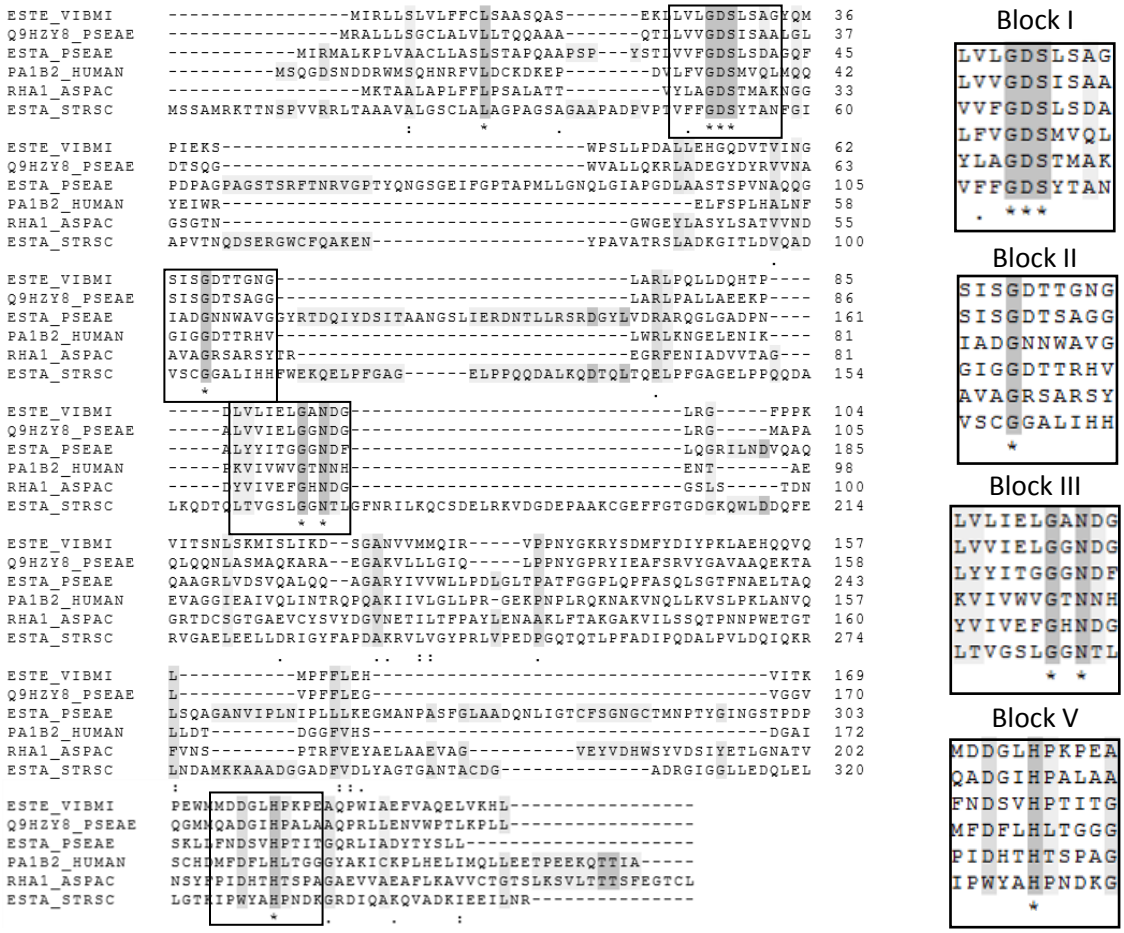


Figure 17: Multiple sequence alignment of (listed from top to bottom) arylesterase from *V. mimicus* [212], TesA from *P. aeruginosa*, esterase EstA from *P. aeruginosa* [28], human platelet-activating factor acetylhydrolase β [214], rhamnogalacturonan acetylesterase from *A. aculeatus* [215] and esterase from *S. scabies* [130]. Sequences containing the GDSL motif (block I), oxyanion hole Gly (Block II) and Asn (Block III) as well as the DXXH motif with catalytic Asp and His are presented enlarged in separate sections on the right.

4.2.2.3. Sequence homology of LipF

In addition to TesA, another enzyme, named **LipF**, is also predicted to belong to the GDSL-hydrolase family. This enzyme displays sequence homology to a lipase/glycerophospholipid-cholesterol acyltransferase (GCAT) from *A. hydrophila* (44% similarity, 27% identity) [216], phospholipase A2/lysophospholipase from *V. parahaemolyticus* (25% identity, 38% similarity) [196], esterase (EstA) from *P. aeruginosa* (26% identity, 36% similarity) [28], phospholipase A (LpPlaA) from *L. pneumophila* (25% identity, 38% similarity) [123] and lithium-tolerant esterase/lipase from *Arabidopsis thaliana (*22% identity, 34 % similarity)[217]. Hence all above listed homologues of LipF are described as a GDSL-hydrolases.

The multiple sequence alignment (Figure 18) of LipF and the homologues revealed strong conservation of the GDSL motif in Block I. Furthermore, the DxxH motif (Block V) and oxyanion hole Asn (Block III) are strongly conserved among all sequences. Unfortunately, the global multiple sequence alignment does not give a consensus sequence for Block II, which includes the oxyanion hole Gly. There is one Gly between Block I and Block III that is conserved in five of six sequences (marked red in Figure 18), but it is not a part of the oxyanion hole in EstA [28, 152] and LpPlaA [123]. Although the global sequence alignment could not detected Block II of LipF the local alignment revealed a consensus sequence for Block II (section Block II in Figure 18) with a strongly conserved Gly residue that was described for LipF homologues as an oxyanion hole residue. Based on this knowledge, we can predict that Ser38, Asp285 and His288 are catalytic triad residues of LipF and additionally that Gly98 and Asn147 are residues of the oxyanion hole. With respect to the functional similarity of LipF to esterase, lipase, phospholipase A, lysophospholipase and even non-hydrolytic enzyme acyltransferase, we can roughly predict that LipF possesses one or more of these activities.

Figure 18: Multiple sequence alignment of (listed from top to bottom) phospholipase A from *Legionella pneumophila* (Q5X2U6_LEGPA) [123], lipase/glycerophospholipid-cholesterol acyltransferase from *Aeromonas hydrophila* (GCAT_AERHY) [216], LipF from *P. aeruginosa* (Q9HUP2_PSEAE), phospholipase A2/lysophospholipase from *Vibrio parahaemolyticus* (HLT_VIBPA) [196], esterase (EstA) from *P. aeruginosa (*ESTA_PSEAE) [28] and lithium-tolerant esterase/lipase from *Arabidopsis thaliana* (LTL1_ARATH)[217]. The sequences containing the GDSL motif (Block I), the oxyanion hole Gly (Block II) and Asn (Block III) as well as the DXXH motif with the catalytic Asp and His are presented enlarged in separate sections. The conserved Gly from Block II are marked in the multiple alignment by the frame.

4.2.2.4. Sequence homology of PlbF

PlbF is annotated in the Pseudomonas Genome Database [19] as a probable lipase due to its homology to the lipase of *Moraxella* sp. According to the Pfam database, it is assigned to a huge family of α/β-hydrolases (PF00561). The crystal structures of 1316 enzymes (Pfam) with an α/β-hydrolase fold are available to date and reveal insights in this common hydrolase fold [129]. Lipases with an α/β-hydrolase fold are characterized by a GXSXG motif around the catalytic Ser [202], which is located in a sharp γ-turn called the “nucleophile elbow” [129]. In addition to the nucleophilic Ser, the catalytic triad Asp and His also have a crucial role in lipase catalysis.

To explore the homology of PlbF to lipases with an α/β-hydrolase fold in more detail, a BLAST search was performed with the PlbF sequence. Unexpectedly, we found 100% sequence homology of PlbF to previously described lipases: Lip3 from *P. aeruginosa* LST-03 [218] and EstA from *P. aeruginosa* 1001 [219, 220]. It should be mentioned that the EstA referred to here is not homologous to the well known outer membrane esterase EstA from *P. aeruginosa* PA01 [28], but these enzymes were given the same name by different authors. EstA and Lip3 were examined from biotechnological point of

view. EstA was described as an esterase (hydrolyses tributyrin) that catalyses production of optically pure (*R*)-β-acetylmercaptoisobutyric acid (RAM) by hydrolysing the corresponding (*R,S*)- β-acetylmercaptoisobutyrate methyl ester [219]. RAM has biotechnological value as an intermediate in the synthesis of pharmaceutically active compounds (Captopril and Alacepril) that are used for the treatment of hypertension and congestive heart failure [221]. Lip3 attracted the attention of authors [218] because it originated from a *P. aeruginosa* LST-03 that tolerates organic solvents. It had already been described that this strain produces organic solvent-resistant lipase, and therefore, the authors examined whether Lip3 is also resistant to organic solvents. Detailed enzymatic and physiological functions of Lip3 and EstA were not discussed in the above mentioned publications.

Furthermore, a BLAST search revealed homology of PlbF to enzymes in which the active sites are structurally well characterised. The sequence alignment of PlbF with those homologues demonstrates strong conservation of catalytic triad residues (Figure 19). Thus, Ser137, Asp286 and His258 emerge as putative catalytic residues of PlbF. BLAST results reveal homology of PlbF exclusively to α/β-hydrolases (Table 13), which were explored in detail with respect to their catalytic properties and physiological functions to learn about PlbF. We have grouped homologues into three physiologically distinct groups: A) lipases from psychotrophic organisms, B) bacterial enzymes that participate in aromatic catabolism, and C) mammalian hydrolases that participate in lipid signal pathways (Table 13). The observed functional homology clearly demonstrates that PlbF is a hydrolase, but prediction as a true lipase (in Pseudomonas Genome Database [19]) is not very reliable. PlbF shares homology to hydrolases that cleave a range of lipids (aryl-esters, monoacyl-glycerol, tributirine, lysophospholipids and phospholipids) and to enzymes that hydrolyse the carbon-carbon (C-C) bonds of acetoin, cholesterol and aromatic compounds. The sequence homology data presented here leave open the possibility that PlbF is not only an esterase, lipase and phospholipase, but also a hydrolase that cleaves C-C bonds. The relevance of the homology of PlbF to the above mentioned three groups (A, B and C) with respect to their physiological functions will be discussed later (Discussion 5.4.1).

A	*Moraxella* sp. TA144 lipase (EC : 3.1.1.3) P24640 [222] *Psychrobacter immobilis* lipase (EC: 3.1.1.3) Q02104 [202] Human hormone sensitive lipase (EC: 3.1.1.79) Q05469 [223]
B	*Pseudomonas resinovorans* meta cleavage compound hydrolase (EC: 3.7.1.8) Q84II3 [224] *Mycobacterium tuberculosis* 2-hydroxy-6-phenylhexa-2,4-dienoic acid hydrolase (EC: 3.7.1.-) P96851 [225] *Pseudomonas fluorescens* 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate hydrolase (EC: 3.7.1.9) P96965 [226] *Pseudomonas putida* 2-hydroxymuconic semialdehyde hydrolases (EC: 3.1.1.-) P23106 [227] *Pseudomonas putida* 2-hydroxy-6-oxo-2,4-heptadienoate hydrolases (EC: 3.1.1.-) P23133 [228] *Ralstonia eutropha* acetyltransferase (EC: 2.3.1.12) P27747 [229]
C	Mouse monoacylglycerol lipase (EC: 3.1.1.23) Q8R2Y0 [230] Mouse lysophospholipase/phospholipase B (EC: 3.1.1.-) Q8VD66 [231]

Table 13: Sequence homologues of PlbF. **A)** lipases from psychrophilic organisms, **B)** bacterial enzymes that participate in aromatic catabolism, and **C)** mammalian hydrolases that participate in lipid signal pathways. For each homologue, the biological source, enzyme name, EC group and UniProtKB accession code are given.

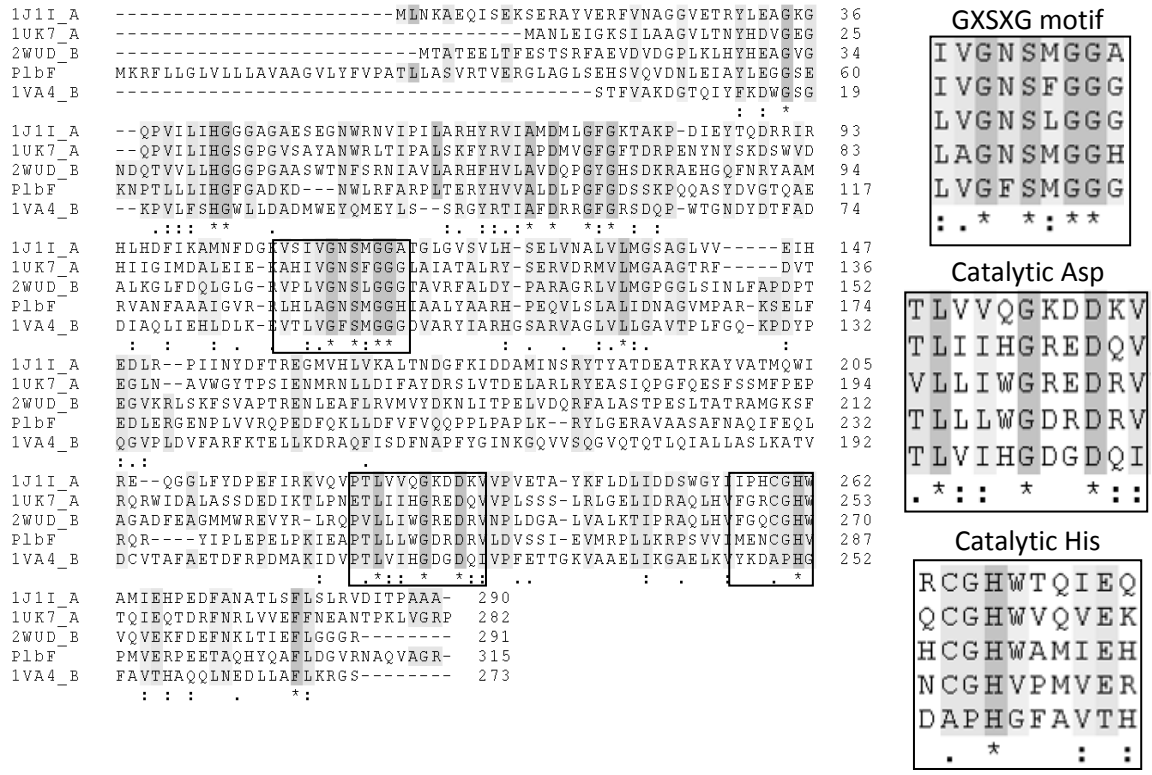


Figure 19: Multiple sequence alignment of *P. resinovorans* hydrolase (1J1I_A, % identity/similarity = 27/44) [224], *P. fluorescens* hydrolase (1UK7_A, % identity/similarity = 24/46) [226], *M. tuberculosis* hydrolase (2WUD_B, % identity/similarity = 26/41) [225], PlbF and *P. fluorescens* arylesterase (1VA4_B, % identity/similarity = 24/41) [232]. Three short sequences with a conserved catalytic Ser in GXSXG pentapeptide, Asp and His are displayed in enlarged sections to the right.

4.2.2.5. Sequence homology of PlpD

PlpD has evoked our attention due to the probability that it is an autotransporter protein with a putative patatin-like phospholipase (PLP) domain. An analysis of 369 bacterial genomes by Banerji *et al.* [89] revealed that pathogenic and symbiotic bacteria, in contrast to non-pathogenic bacteria, encode a much higher number of PLP enzymes. In the same study, it was stressed that intracellular bacterial pathogens (*L. pneumophila*, *R. prowazekii* and *M. tuberculosis*) are species with high densities of PLP-encoding genes. Indeed, PLP enzymes of *P. aeruginosa* (ExoU) [106, 115] and *L. pneumophila* (PatD) [233] have been proven to be virulence determinants of these human pathogens. PatD and ExoU exhibit phospholipase A activity. PatD is required for replication of *L. pneumophila* in *A. castellanii* amoebae and human macrophages thus contributing to the *L. pneumophila* virulence [233].

The mechanism of ExoU pathogenicity is comprehensively understood (Chapter 1.1.3) in view of the fact that it was the first characterised bacterial PLP. Therefore, its roll in acute pneumonia [116], pneumosepsis [118] and infections of airway cells [117] caused by *P. aeruginosa* is well established [63]. ExoU is not encoded by all *P. aeruginosa* strains (or by *P. aeruginosa* PA01); therefore, it was meaningful to look for ExoU homologues in *P. aeruginosa* PA01 genome. PlpD emerged in a BLAST search as a homologue of ExoU with 40% sequence similarity and 25% sequence identity on the sequence spanning the PLP domain.

The Pfam database predicts three domains in PlpD: the PLP domain (aa 27–220), the surface agent variable number repeat (saVNR) domain (aa 333–400), and the bacterial surface antigen domain (Bsa) (aa 438–728). The multi-domain organisation of PlpD is in line with the observation of Banerji *et al.* [89] that many PLPs are two-or-more-domain proteins. This observation could be supported by our finding that 23% of the members of the PLP family (PF01734) indeed possess at least two domains, according to the Pfam database. Bacterial patatin-like domains (responsible for enzymatic activity of patatins) contain active site consisting of a catalytic dyad (Ser-Asp) instead of the catalytic triad typical for the serine hydrolase family [234]. Four short conserved sequences of homology (named Blocks I – IV) are conserved among bacterial PLPs [234]. The nucleophilic serine and catalytic aspartate are part of Blocks II and IV, respectively. In Block I, a conserved arginine or lysine forming the oxyanion hole is located beside a stretch of mostly three glycine residues. Block III contains conserved serine and proline residues, which may have an important structural roll together with the conserved proline from Block IV [234]. All four sequences of homology characteristic of PLP proteins could be identified in PlpD (Figure 20). Therefore, we predicted that the active site of PlpD consists of Ser60 and Asp207, which form the catalytic dyad, and Arg35, which stabilises the oxyanion intermediate during hydrolysis. Furthermore, amino acids with a putative structural role in PLPs that are conserved in Block III (Ser and Pro) and Block IV (Pro) align to Ser188 and Pro192 (Block III) and Pro215 (Block IV) of PlpD (Figure 20).

Figure 20: Multiple sequence alignment of PlpD and its closest characterised homologues: neuropathy target esterase from *Homo sapiens* (PLPL6_HUMAN) [235], lysophospholipase NTE1 from *Saccharomyces cerevisiae* (NTE1_YEAST) [236], neuropathy target esterase from *Mus musculus* (PLPL6_MOUSE) [237], patatin-like phospholipase from *Rattus norvegicus* (PLPL7_RAT) [238], phospholipase from *P. aeruginosa* PA14 (ExoU) [239]. PLP domains of each enzyme were used in the alignment as they are defined in Pfam database.

A BLAST search revealed homology of PlpD exclusively to the eukaryotic enzymes (Figure 20) in keeping with observations that bacterial PLPs differ strikingly from all known bacterial hydrolase groups and are more related to the eukaryotic PLPs [89]. All PlpD homologues detected by the sequence homology search possess esterase activity) [235, 236, 237, 238, 239] but only human NTE and yeast NTE1 also possess lysophospholipase and phospholipase A2 [235, 235, 240] activities. It has been described that ExoU is able to hydrolyse phospholipids in a lysophospholipase- and phospholipase A2-dependent manner [107]. Taking the conservation of the active site and structurally important residues in PlpD and its homologues (Figure 20) together with the experimentally demonstrated hydrolytic activity of PlpD homologues, it is not unlikely that PlpD could be an esterase, phospholipase A2 or lysophospholipase.

Another two domains detected in PlpD (surface antigen variable number repeat (VNR) and bacterial surface antigen domains) are located in the carboxy-terminal part of the enzyme, and these domains are not responsible for enzymatic activity. The surface antigen VNR domain is a small domain (average 78 aa) with a conserved GY/F dipeptide at positions 52-53 in the consensus sequence (Figure 21). This domain is almost exclusively followed by a bacterial surface antigen domain [184]. Indeed, among 5265 proteins possessing the surface antigen VNR domain, according to the Pfam database, more than 97% of members have a bacterial surface antigen domain at their carboxy-terminus. This particular architecture was noted in the outer membrane proteins from pathogenic bacteria, such as the D15 protein from *H. influenza* [241] and *H. ducreyi* [242], the OMA87 protein form *P. multocida* [243] or the OMP85 protein from *N. meningitidis* [244, 245] and *N. gonnorhoeae* [246]. For all proteins mentioned above, a role in pathogenesis has been suggested because they have been proven to be key antigens of these pathogens. A BLAST search with the PlpD sequence resulted with identification of only a few proteins with homology in the part of carboxy terminal sequence, which encodes for putative surface antigen VNR and bacterial surface antigen domains. All of the homologues were uncharacterised proteins with the exception of the Omp85 protein from *N. meningitidis* [244, 245], which shares 33% similarity and 20% identity with PlpD. Members of the Omp85 family are classified as a part of two partner secretion system.

In our recently published work [247], we demonstrated that the bacterial surface antigen domain of PlpD has a function of an autotransporter. Thereby, we recognised the hybrid mechanism of two partner secretion (Type Vb) and autotransporter secretion (Types Va and Vc) as a novel Type Vd secretion system.

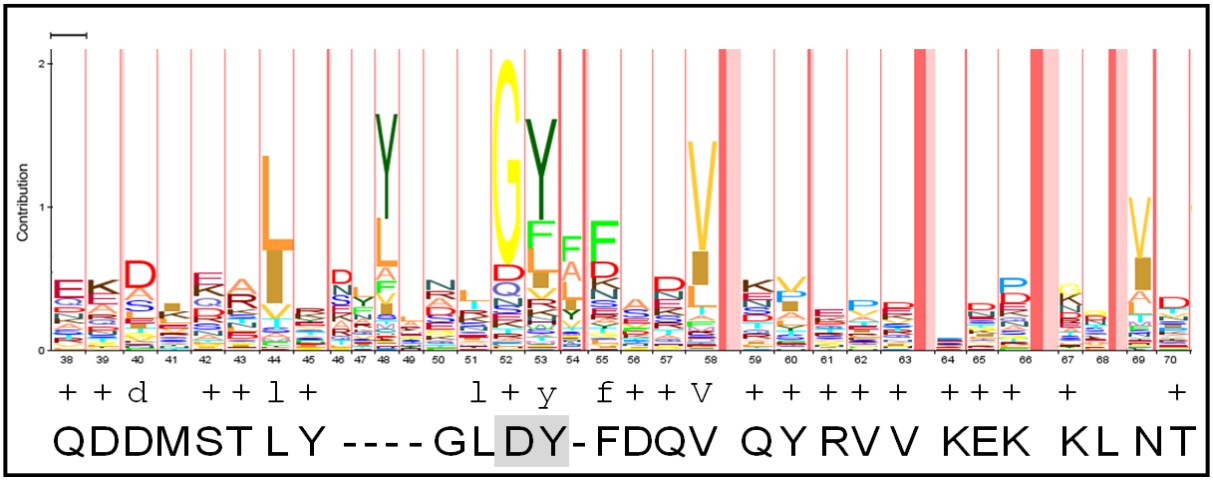


Figure 21: Sequence alignment of the consensus sequence of the surface antigen VNR domain (positions 38 - 70) and PlpD (aa 369 - 396). The consensus of the surface antigen VNR domain is presented by partial logo (multicoloured upper of alignment) [248]. The PlpD sequence is presented at the bottom of the alignment in black. The characteristic dipeptide conserved in surface antigen VNR domain of PlpD is displayed on a grey background. The relative height of each letter in the partial logo stack is proportional to its frequency at the position (for example, G52 is more conserved than D40). If the position in the stack is broader, it is more conserved in the family (position 55 is more conserved in the family than position 48). The total width of the red-shaded (dark + light) line between two positions represents the expected number of inserted letters between these two positions (much fewer letters are expected to be inserted between positions 52 and 53 than between positions 58 and 59). The width of dark red part of the line represents the probability that at least one letter is inserted (it is much more probable that at least one letter is inserted between positions 63 and 64 than between positions 54 and 55).

4.2.2.6. Sequence homology of PlaB

PlaB is one of two members of the phospho-lipolytic system that is annotated as a hypothetical protein in the Pseudomonas Genome Database [19]. However, performing a BLAST search, we identified that PlaB shares sequence homology (25% identity and 39% similarity) to *L. pneumophila* phospholipase A [198]. Analogously with described PLA from *L. pneumophila* (PlaB), we also named the *P. aeruginosa* homologue PlaB. In the following text, the abbreviation PlaB will be used for the *P. aeruginosa* enzyme, and LpPlaB will be used for the *L. pneumophila* enzyme.

A search of the Pfam database with the PlaB sequence to find conserved domains in PlaB did not yield any significant results. Furthermore, neither of the two typical motifs that surround the catalytic Ser (GXSXG or GDSL motive) in most hydrolases was found in the PlaB sequence. However, a BLAST search revealed homology between PlaB and hypothetical enzymes and putative phospholipases. Multiple sequence alignment (Figure 22) revealed a notably higher homology of PlaB homologues to the amino terminal part of PlaB (aa 1-270) in comparison to its carboxy terminal part. Recently, the active site of LpPlaB was annotated [249], giving insight into the essential catalytic residues of the novel lipase family proposed by authors. Thus, the classical catalytic triad of serine hydrolyses, which is composed of Ser85, Asp203 and His251, was recognised in LpPlaB. Furthermore, His7 and Ser129, which are required for hydrolysis of dipalmitoyl phosphatidylcholine (the phospholipase B substrate) but not a dipalmitoyl phosphatidylglycerol (the lipase substrate) emerged as residues that determine the substrate specificity of LpPlaB. The novel feature of the lipolytic family

represented by LpPlaB is the presence of a catalytically active Ser in the T/VHSXG pentapeptide, which is similar to the typical lipase motif, GXSXG, but appears to have a different role in catalysis. It was shown experimentally that mutation of the first aa of the $THS_{85}TG$ pentapeptide to Gly (generating a GXSXG motif) in LpPlaB abolished its activity by 95%, but mutation of the same Thr to Val, which is found in other members of this family at the same position, influenced only the substrate specificity of LpPlaB. Therefore, the importance of first residue in conserved pentapeptide for effective hydrolysis of this group of enzymes was proposed. All catalytic amino acids are strongly conserved between PlaB and its homologues, which allow us to propose that Ser79, Asp196 and His244 are the catalytic triad residues of PlaB. According to the sequence alignment, His9 and Ser121 of PlaB are the residues that may influence substrate specificity of PlaB.

Figure 22: Multiple sequence alignment of PlaB and its homologues: *L. pneumophila* phospholipase A (LpPlaB) [198], *Marinobacter algicola* uncharacterized protein (A6EVY1_9ALTE), *Shewanella pealeana* putative phospholipase (A8GZA7_SHEPA), *Shewanella loihica* putative phospholipase

(A3Q9J5_SHELP), *Mariprofundus ferrooxydans* putative phospholipase (Q0EXH0_9PROT) and *Legionella drancourtii* putative phospholipase (C6N4I1_9GAMM). The conserved sequence around the catalytic triad residues is marked by a black frame. The conserved His and Ser with a role in substrate specificity are highlighted in red. Ten amino-terminal amino acids of *Legionella drancourtii* putative phospholipase were removed from the alignment for simplicity purpose.

4.2.2.7. Sequence homologies of PldB and PldC

The genes PA2155 and PA4339 encode for previously uncharacterized enzymes predicted to be phospholipase Ds; therefore, we named them PldB and PldC, respectively. Two phospholipase D active site motifs (**H**X**K**X_4**D**X_6**G**) conserved among members of PLDc Pfam family (PF00614) are present in both proteins. It is not unexpected to have two identical PLA motifs per one protein molecule because it was demonstrated for other PLDs that two histidines of **H**X**K**X_4**D**X_6**G** motif form a single catalytic site. Other members of this family are cardiolipin and phosphatidylserine synthetases and bacterial, viral, mammalian and plant PLDs. These enzymes are involved in phospholipid remodelling, and mostly, they specifically hydrolyse phosphatidylcholine, but PLDs that hydrolyse phosphatidylinositol have been described [26].

A BLAST search with the PldB and PldC sequences identified homology to *B. pseudofirmus* cardiolipin synthetase (BpCLS) [250] and another putative and uncharacterised CLS. BpCLS shares 29% sequence identity (50% similarity) to PldB and 29% sequence identity (50% similarity) to PldC. The homology of PLD and CLS was already mentioned above, and it could be additionally strengthened by the finding that PLD from cabbage also possesses cardiolipin synthetase activity [251]. Its dual activity (PLD and CLS) was explained by a proposed catalytic mechanism that involves first cleaving phosphatidyl glycerol to phosphatidate and glycerol (PLD activity) followed by condensation of phosphatidate with phosphatidyl glycerol yielding cardiolipin (CLS activity) [251]. It is described that the sequences of PLDs are not highly conserved outside of the conserved active site motifs. Therefore, the multiple sequence alignment (Figure 23) included previously described PLDs with much lower sequence homologies to PldB and PldC, namely PLD from *P. aeruginosa* (PldA) [26], CLS from *E. coli* (EcCLS) [252] and PLD from *Streptomyces* sp. (SsPLD) [253]. PldA is the only described PLD in *P. aeruginosa*, EcCLS is known as the most extensively studied bacterial enzyme belonging to the PLD family, and SsPLD is one of four PLD family members with a solved 3D structure [253]. Another three members of PLD family, Nuc (from *S. typhimurium*) [254], Tdp1 (from *H. sapiens*) [255] and Ymt (from *Y. pestis*) [256], are able to bind and cleave phosphodiester moieties (similar to PLDs or CLSes), but their functions are very unlike PLD. Nuc is an endonuclease that hydrolyses DNA, Tdp1 hydrolyses protein-DNA complexes, and the natural substrate of Ymt is not known to date [253]. Therefore, these enzymes were not included in the sequence alignment. Although the global sequence homologies of the chosen enzymes were low, two short sequences (Block I and II) containing the characteristic HXKX_4DX_6G motif were highly conserved among them (Figure 23).

In an comprehensive crystallographic study of SsPLD, it was demonstrated that His170, His448, Lys172, Lys450, Asn187, Asn465, Asp202 and Asp473 belong to the active site of this enzyme [253]. Therefore we could annotate putative active site residues of PldB and PldC from the sequence alignment. In Figure 23, it is visible that active site histidines and lysines correspond to His and Lys from the HXKX_4DX_6G motif but that the strongly conserved Asp from the HXKX_4DX_6G motif is not a part of the active site of PLD. The active site Asp is located downstream of the HXKX_4DX_6G sequence,

at a position of +20 in Block I and +11 in Block II relative to the Gly of the $HXKX_4DX_6G$ motif. Furthermore, two active site Asn residues are located in the vicinity of the $HXKX_4DX_6G$ motif, at a position +2 relative to the Gly of the $HXKX_4DX_6G$ motif. One Asn (from Block II) is conserved among all six enzymes, but the Asn from Block I is conserved in four of six enzymes (in PldA is replaced by Asp, and in PldC, there is a Gly at this position). Despite their important role for enzyme catalysis, it seems that active site Asp and Asn are not as strictly conserved in the sequence alignment as His and Lys.

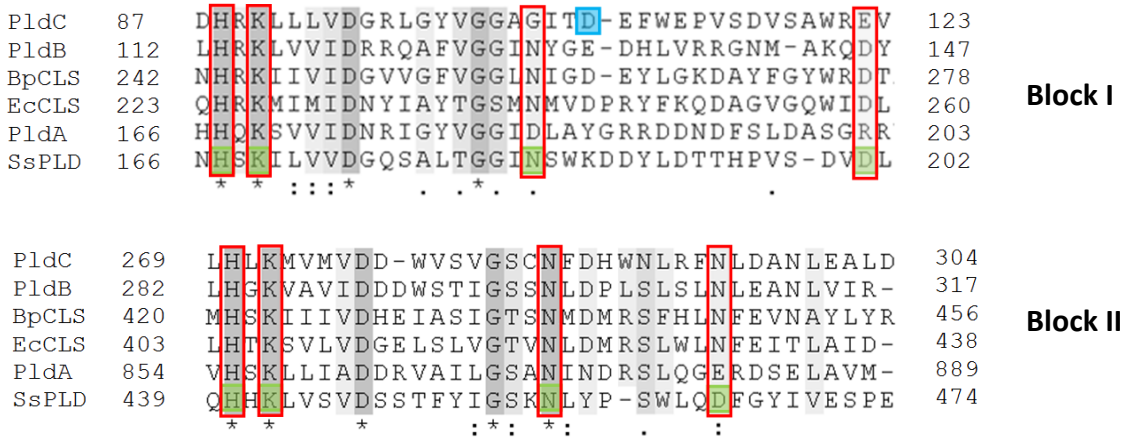


Figure 23: Multiple sequence alignment of PldC, PldB, PldA [26], BpCLS [250], SsPLD [253] and EcCLS [252]. Short sequences (Blocks I and II) around the active site residues are shown due to the low global sequence homology. Numbers preceding and following the blocks represent the position of the first and last amino acid in the alignment. Residues marked by a red window are demonstrated and putative active site residues conserved in the sequence alignment. The putative active site Asp of PldC is highlighted in blue. The catalytic residues of SsPLD as annotated from 3D structure are highlighted in green.

It was already mentioned that Gly105 is present in the sequence of PldC at the position of the active site Asn in Block I. The side chain of Gly does not possesses the same physical-chemical properties as Asn, and consequently, it cannot form stabilising H-bond to the His residue in the active site [253]. Therefore, is very unlikely that Gly105 is an active site residue of PldC. Looking to the sequence of PldC in the vicinity of the conserved $HXKX_4DX_6G$ motif, no Asn was identified. However, Asp108 is found three positions downstream of Gly105, and this residue may serve as a catalytic residue. It was already observed that Asp replaces the conserved active site Asn in Block I of PldA (Figure 23). Generally, three dimensional structures are evolutionary better conserved than sequences [257], we tested whether Asp108 may be structurally conserved with Asn187 of SsPLD. Therefore, the 3D structure of PlaC was modelled *in silico* (on Phyre web server) [187] using the structure of SsPLD as a template (data not shown). The alignment of the PldC model with the SsPLD structure (PDB entry 1V0W) showed overlapping of Gly105 (but not Asp108) of PldC with Asn187 of SsPLD. Therefore, the question of which residue functionally replaces the catalytic Asn in Block I of PldC still remains.

The prediction of two catalytic Asp in PldB and PldC was challenging because clear data about catalytic Asp exists only for SsPLD. The catalytic Asp from Block I was conserved among four sequences, among them is PldB (Asp146). Furthermore, Glu122 of PldC was present in the alignment at the position of the conserved Asp. It could form an H-bond to the catalytic His as Asp202 of SsPLD does due to the same physical-chemical properties of Asp and Glu. The sequence alignment of Block II revealed the presence of Asn in the sequence of PldB (Asn309) and PldC (Asn295) at the position of

the conserved Asp. Both Asn and Asp can form an H-bond with the side chain carboxamide and carboxy group, respectively. Therefore, it is not unlikely that Asn takes over the role of Asp in the active sites of PldB and PldC. Indeed, it was observed that in the crystal structure of Tdp1 (a member of the PLD family), Gln (Asn and Gln have both an amido group in the side chain) takes over the role of the catalytic Asp from Block I [255].

Summing together, we can propose that His113, His283, Lys115, Lys285, Asn130, Asn300, Asp146 and Asn309 are the active site residues of PldB and that His88, His270, Lys90, Lys272, Asn295, Glu122 and Asn287 are the active site residues of PldC. Even though the catalytic amino acids of PldB and PldC are predicted with high reliability (except catalytic Asn from block I of PldC), their enzymatic functions cannot be predicted so confidently. Both enzymes could be PLD or CLS, but the possibility that they cleave a variety of natural substrates, for instance DNA or protein-DNA complexes, like other members of the PLD family, such as endonuclease (Nuc) [254] and phosphodiesterase (Tdp1) [255], is not excluded.

4.2.2.8. Sequence homology of EstC

EstC is classified according to the Pfam database as a member of the esterase family (PF00756), which belongs to the group of α/β-hydrolases. A BLAST search revealed its high homology (57-75% sequence similarity) to four esterases with solved 3D structures (Figure 24) and additionally to previously described esterases (without known 3D structures) from *S. cerevisiae* (A1AXZ2) [258], *A. thaliana* (Q8LAS8) [259] and *P. denitrificans* (P40363) [260]. Interestingly, all of those esterases possess hydrolytic activity against S-formylglutathione, and therefore, they are also called S-formylglutathione hydrolases (SFGH, EC 3.1.2.12). Previously, it was demonstrated that SFGHs are serine hydrolases with a Ser-Asp-His catalytic triad and an α/β-hydrolase fold [261]. Sequence alignment (Figure 24) shows strongly conserved catalytic motifs characteristic for SFGH enzymes. The oxyanion hole Leu is located close to the amino terminal end of the protein in the conserved LSG**L**TC motif (bold stressed Leu is a part of oxyanion hole). Nucleophilic Ser and oxyanion hole Met are present in the GH**SM**G motif as a Ser-Met dipeptide. The oxyanion hole residue following the catalytic Ser makes this group of enzymes different from typical esterase/lipase families, which have a separate GXSXG motif and a motif that contains the oxyanion hole residue. The catalytic triad His and Asp are present downstream of the catalytic Ser in the conserved D**H**SYYF and **D**XF motifs, respectively [261].

The data presented here allow us to predict that Ser152, His261, Asp228, Leu54 and Met153 are the active site residues of EstC. Because esterase activity and S-formylglutathione hydrolase activity were described for all homologues of EstC, it is not too speculative to predict that EstC is an esterase or S-formylglutathione hydrolase.

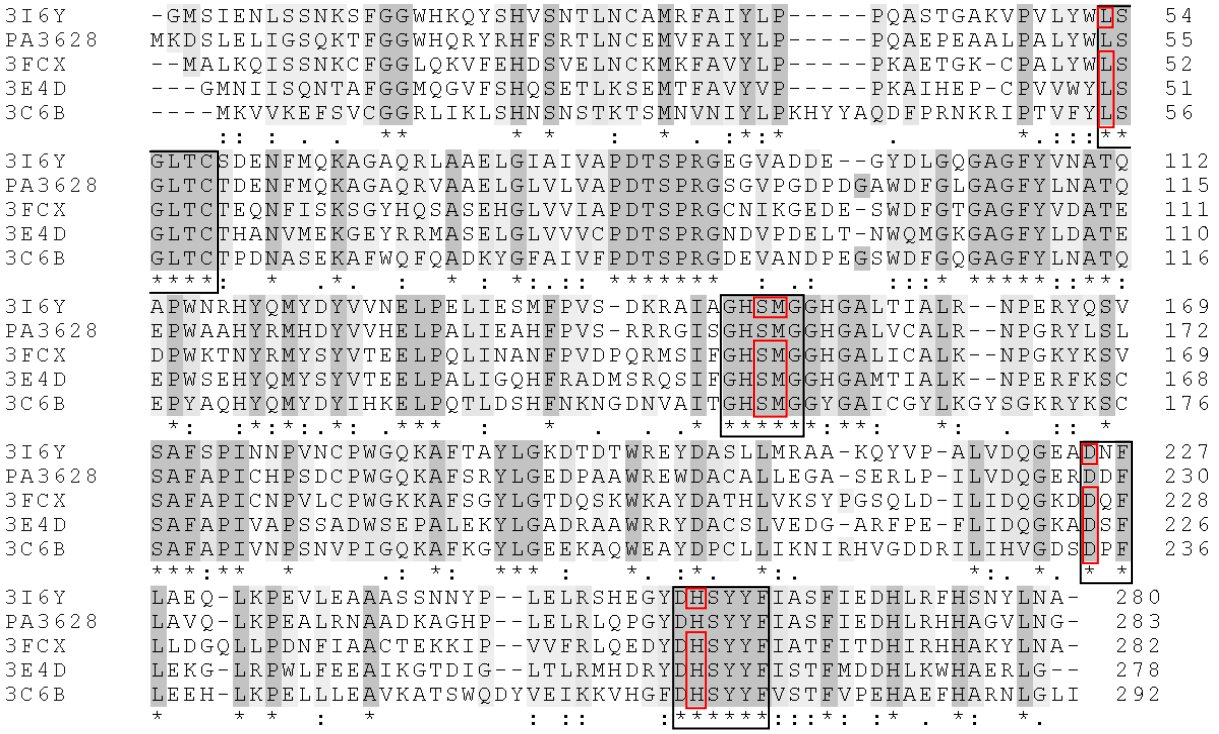


Figure 24: Multiple sequence alignment of EstC (PA3628) and its homologues with solved 3D structure: *O. antarctica* esterase (3I6Y, 65/75% seq. identity/similarity), human esterase D (3FCX, 56/72% seq. identity/similarity) [262], *A. tumefaciens* Esterase D (3E4D, 49/61% seq. identity/similarity) [261], and *S. cerevisiae* thioesterase (3C6B, 41/57% seq. identity/similarity) [263]. The sequence motifs containing active site residues are marked by black frame. The active site residues inferred from the 3D structures are highlighted in red.

4.2.2.9. Sequence homology of EstB

EstB is classified in the Pfam database as an α/β-hydrolase (PF07859), which was confirmed by the BLAST results. A homology search revealed that EstB is related to the hormone sensitive lipase (HSL) family (EC 3.1.1.79) [264, 265], which is reported to include true lipases, carboxylesterases and amidases [266]. To predict the active site of EstB, its primary sequence was aligned with the sequences of homologous proteins in which 3D arrangement of the catalytic residues is known. Thus, HSL esterases from *B. subtilis* and *A. acidocaldarius* together with three HSL esterases from metagenome were aligned (Figure 25) [267, 268, 269, 270, 271]. As all EstB homologues are previously described HSL enzymes, the multiple sequence alignment provides insights in conserved regions characteristic for the HSL family. The catalytic triad (Ser-His-Asp/Glu) typical for the Ser-hydrolase family is conserved in alignment. The nucleophilic Ser is present in the conserved GXSXG pentapeptide, and the catalytic triad His and Glu are located between the GXSXG motif and the carboxy terminal end of enzyme.

In the primary structure, the oxyanion hole residue, mostly Ala, is located immediately after the catalytic Ser. The conserved HG**GG** motif located between the amino terminal end and the GXSXG motif is composed of two oxyanion hole Gly residues (bold stressed GG in HG**GG** motif). Based on the sequence alignment, we can predict Ser150, Glu243 and His273 are the catalytic triad of EstB. Furthermore, Gly82, Ala83 and Ala151 are the putative oxyanion hole residues of EstB. It is worth noting that Ala is present in the alignment at the position of the second conserved oxyanion hole Gly

in the HGGG motif. In addition to homology to the already mentioned HSL enzymes, EstB possesses significant homology (30-32% sequence identity) to mouse [272], human [223], rat [273] and pig [274] HSLs, which show high sequence identity (more than 85%) mutually. Furthermore EstB is homologous to other eukaryotic hydrolases, namely acetyl monoalkylglycerol ether hydrolase from mouse [275] and its orthologous cholesterol ester hydrolase from human [276]. However, it was previously published that many HSLs hydrolyse cholesterol esters in addition to mono-, di- and triacylglycerol [276, 277]. The bacterial homologues of EstB are *E. coli* esterase [278] and lipase (lip2) from *Moraxella* TA144 [279]. Both belong to the HSL family. The high sequence homology of EstB with esterases and lipases that belong to the HSL family allows prediction of a putative esterase and lipase function of EstB. Furthermore, many HSL enzymes posses cholesterol ester hydrolase activity, and it is tempting to speculate that EstB could hydrolyse cholesterol esters.

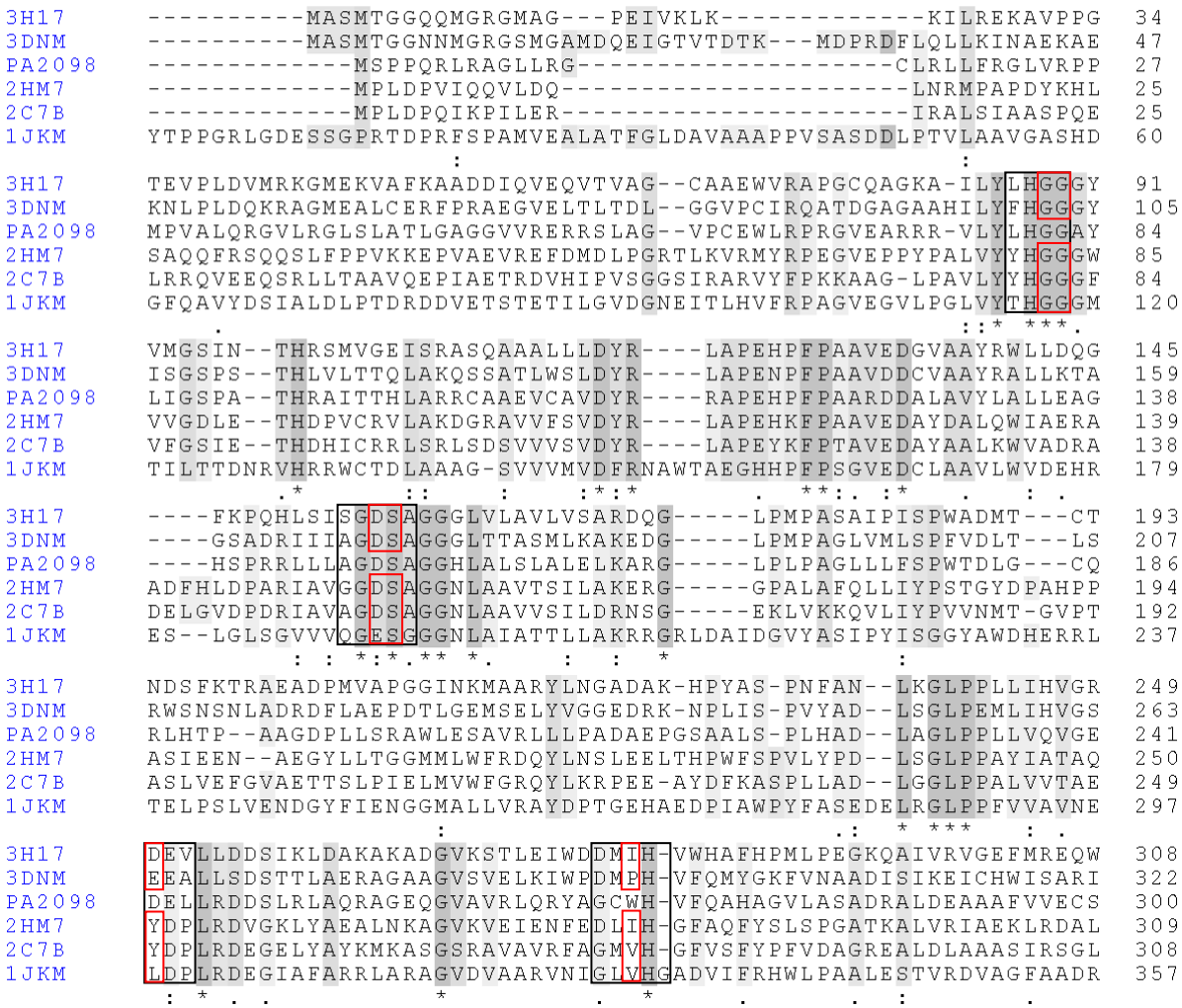


Figure 25: Multiple sequence alignment of *P. aeruginosa* EstB (PA2098) and its homologues with solved 3D structure: 3DNM [267], 3 h1Z [268] and 2C7B [269] esterase from the metagenome library; 1JKM, esterase from *B. subtilis* [270]; and 2 hM7, esterase from *A. acidocaldarius* [271]. The sequence motifs containing the active site residues are marked by black frames. The active site residues annotated from the 3D structures are marked in red.

Protein organisation and prediction of the active site residues	Predicted enzymatic function
 Ser78, Tyr167	Esterase, β-Lactamase
 Ser150, Glu243, His273, Gly82, Ala83, Ala151	Cholesterol esterase Lipases (HSL)
 His113, Lys115, Asn130, Asp146, His283, Lys285, Asn300, Asn309	Phospholipase D, Cardiolipin synthetase
 Ser30, Asp177, His180, Gly67, Asn96	Esterase, LysoPLA Thioesterase, Protease
 Ser79, Asp196, His244	Phospholipase A
 Ser137, Asp286, His258	Lipase, Lysophospholipase, C-C hydrolase
 Arg, 35, Ser60, Asp207	Lipase, Phospholipase A2 Lysophospholipase
 Ser152, His261, Asp228, Leu54, Met153	Esterase S-formylglutatione hydrolases
 His88, Lys90, Asp108, Asp122, His270, Lys272, Asn287, Asn295	Phospholipase D, Cardiolipin synthetase
 Ser38, Asp285, His288, Gly98, Asn147	Lipase, LysoPLA Phospholipase A, Acyltransferase
	

Figure 26: A functional model of phospho-lipolytic system built according to the predictions of enzymatic functions. In the model, protein sequences are presented as a diagram with putative domains displayed in brown colour and active site residues marked by red pins and additionally listed under the diagram. The models were created with the online available MyDomains tool [186]. All enzymes are predicted as one domain proteins with the exception of PlpD, for which PLP (patatin-like), saVNR (surface antigen variable number repeat) and BSA (bacterial surface antigen) domains were predicted.

Summary

4.2.2 Sequence homology and prediction of active sites of phospho-lipolytic enzymes

To summarize the homology data obtained about members of the phospho-lipolytic system, a functional model predicting molecular organisations, active sites and enzymatic functions was introduced (Figure 26). The model gathers 14 different enzyme classes to which phospho-lipolytic enzymes might belong that indicate the complexity of the phospho-lipolytic system. Moreover, the major potential of the enzymes chosen here is underlined by the knowledge that members of these enzyme families have important cellular functions in other microorganisms and eukaryotes. However, the *in silico* data presented here should be experimentally tested. It may be mentioned that in keeping with our aim, candidates with putative phospholipase A and B and lysophospholipase activity will be our particular focus.

4.2.3. *In silico* prediction of cellular localisation of phospho-lipolytic enzymes

Functional characterisation of proteins in the context of cellular compartments is one of fundamental goals in cell biology. Many *in silico* algorithms are currently available for predicting bacterial protein subcellular localisation. These methods are based on the detection of signal peptides recognised during the translocation of proteins through the membrane, or hydrophobic helices and outer membrane protein motifs spanning through the membrane and serving as an anchor [280]. Recently was a comparative study of *in silico* and experimental localisation methods demonstrated that the approach of utilizing multiple prediction methods is likely to provide significantly better results in comparison to single method predictions [281 EN 262]. Guided by this idea, three different methods, namely the Hidden Markov Model (HHM), neural network (NN) and weight matrix methods, were employed in the prediction of signal peptide in phospho-lipolytic enzymes using Signal P-HMM [188], Signal P-NN [188] and SigCleave [282] programs, respectively [283]. Transmembrane helices and their topologies were predicted using MEMSAT3 [191], TMpred [284] and Toppred [193], tools available online. The outer membrane motif typical for *Gram-negative* bacteria was predicted by the pSORTb [189] web tool.

To discriminate signal peptides from non-signal peptides, a D-score output from SignalP-NN higher than 0.5, a C-score output from SignalP-HMM higher than 0.95 and a SigCleave score higher than 5.50 were used. For discrimination of transmembrane helices, scores lower than -5, higher than 500 and higher than 0.8 were used from the MEMSAT3, TMpred and Toppred predictors respectively. The elements (signal peptides and transmembrane helices) that were predicted by the consensus of at least two predictors were considered to be a significant result, and only those elements will be discussed further. Furthermore, localisation of transmembrane helices was examined in the context of protein domain organization. Transmembrane helices that spanned the putative catalytic domain were considered to be false positive results.

An outer membrane motif was only predicted for one phospho-lipolytic enzyme, namely PlpD, which is in keeping with the Pfam database prediction of carboxy terminal bacterial surface antigen domain forming β-barrel structure known to be inserted in the outer membrane. The signal peptide was also

predicted for PlpD, so it is likely that PlpD is first translocated through the inner membrane followed by insertion of C-terminal domain in to the bacterial outer membrane.

In general, the results of prediction of signal peptides were identical using all three predictors (Table 14). In addition to PlpD, signal peptide was predicted in TesA, PlbF and LipF, so it is likely that those proteins are periplasmic or extracellular proteins. Combining the transmembrane helix and signal peptide prediction data of LipF, it is worth noting that the prediction of transmembrane helix (aa 5-29) and signal peptide (aa 1-20) span the same amino terminal protein sequence. Therefore, it is not clear if LipF is a membrane, periplasmic or extracellular protein. However, the agreement in the predicted topology of the LipF transmembrane helix by the three different predictors hints that LipF could be anchored to the membrane by its uncleavable amino terminal signal sequence. It is not likely that TesA and PlbF contain a transmembrane helix (although some transmembrane helices are predicted for them, but only with one predictor), so these enzymes are putative periplasmic or extracellular proteins.

In the transmembrane helix prediction data (Table 15), it is noted that a transmembrane helix was predicted for each protein. However, consensus of at least two predictors only exists for three enzymes (LipF, PldB and PldC). As discussed above, LipF is a protein with predicted uncleavable signal peptide which could serve as a membrane anchor. PldB and PldC do not have putative signal peptides, so it is assumed that they are anchored to the bacterial inner membrane. For PldB, the transmembrane helix is predicted to be in the carboxy terminal portion, but for PldC, it is predicted to be located in the amino terminal portion. Furthermore, prediction of transmembrane helix topology for PldB differs with respect to two predictors. TMpred predicted the N-terminus, inside (cytoplasmic side), and Toppred predicted the N-terminus, outside (periplasmic side), relative to the inner membrane. Therefore, it is not possible to predict whether the catalytic domain of PldB is located on the cytoplasmic or periplasmic side of the inner membrane. Both predictors predicted transmembrane helix topology for PldC with a consensus of carboxy-terminal (catalytic) domain located on the periplasmic side of the inner membrane.

PlaK and EstC do not have predicted signal peptides or transmembrane helices, so it is likely that these two enzymes are cytoplasmic proteins. The last two phosphor-lipolytic enzymes, EstB and PlaB, do not have a putative signal peptide, but transmembrane helix prediction revealed a putative transmembrane helix in each protein by one predictor. As the requirement for consensus of two predictors for the same transmembrane helix was not fulfilled, we tested both sequences using a fourth predictor (HMMTOP [285]). HMMTOP did not predict any transmembrane helix in either protein, so EstB and PlaB were classified as putative cytoplasmic proteins with low possibility to be membrane proteins.

Table 14: Prediction of signal peptides and their cleavage sites. Three predictors were used: Signal P-HMM, Signal P-NN, and SigCleave. The other members of the phospho-lipolytic system do not have putative signal peptides.

Enzyme Name	Prediction of signal peptide length		
	Signal P-HMM	Signal P-NN	SigCleave
TesA	21	21	21
PlbF	28	28	15
PlpD	18	18	18
LipF	20	20	20

Table 15: Prediction of transmembrane helices in phospho-lipolytic enzymes. Topologies of predicted transmembrane helices are annotated with **in-X/Y-out** or **out-X/Y-in**, where **X** and **Y** represent the first and last amino acid spanning the central transmembrane helix segment and **in-** or **out-** indicate the location of terminal amino acid inside or outside of the membrane. A colour code was used to annotate the reliability of the prediction. TMH predicted to be localised in putative catalytic domain are marked in red, TMH predicted by only one predictor are marked in white, and TMH predicted by more than one predictor are green. ND = not defined.

Enzyme Name	Putative catalytic domain / aa	Prediction of transmembrane helix		
		MEMSAT3	TMpred	Toppred
PlaK	25 – 388	in-61/85-out	in-73/89-out	in-72/93-out
EstB	76 – 277	out-231/250-in	out-18/34-in	out-165/185-in
			in-251/269-out	
PldB	1 – 340	in-61/79-out	in-348/370-out	out-353/373-in
TesA	24 – 193	out-120/142-in	in-71/89-out	in-1/21-out
PlaB	1 – 270	out-44/63-in	out-189/207-in	out-363/383-in
PlbF	89 – 302	out-245/263-in	in-65/83-out	in-4/24-out
EstC	25 – 276	out-61/84-in	ND	in-68/88-out
PldC	60 – 300	out-276/300-in	in-39/59-out	in-40/60-out
LipF	21 – 301	in-6/29-out	in-7/26-out	in-5/25-out

Summary:

4.2.3 *In silico* prediction of cellular localisation of phospho-lipolytic enzymes

Based on the prediction data for cellular localization obtained by numerous *in silico* methods, a localisation model of the phospho-lipolytic system is shown in Figure 27. Four enzymes (PlaK, EstB, PlaB and EstC) are putative cytoplasmic proteins, although EstB and PlaB could be membrane proteins to. Four proteins (TesA, PlbF, PlpD and LipF) are putative extracytoplasmic enzymes. However, LipF and PlbF were also predicted to be inner membrane proteins, and PlpD is putative outer membrane protein. Only PldC is predicted to be exclusively associated with inner membranes with the catalytic domain predicted to be localised in the periplasm.

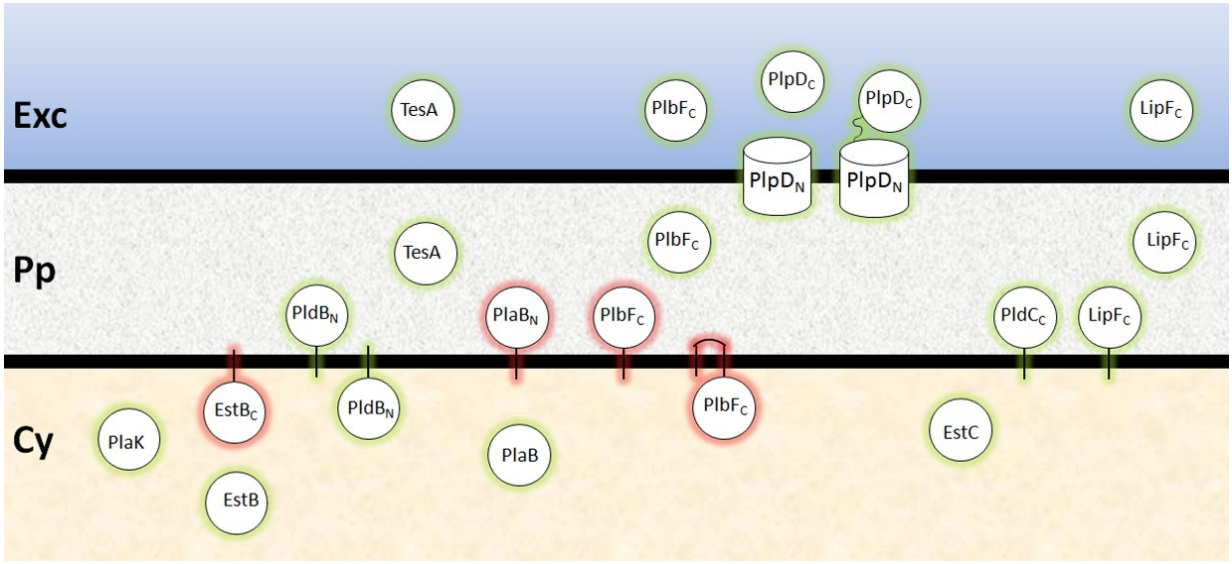


Figure 27: Localisation model of the phospho-lipolytic system proposed according to the results of *in silico* analyses. For some enzymes, various localisation predictions were obtained, so more than one localisation is proposed for them. N and C in subscript next to the enzyme abbreviation indicate N-terminal and C-terminal domains respectively. The colour of each graphic indicates the reliability of the proposed model: green = reliable, red = less reliable. Cy, cytoplasm; Pp, periplasm; Exc, extracellular space.

4.3. Molecular cloning, expression, purification and biochemical characterisation of phospho-lipolytic enzymes

4.3.1. Molecular cloning of genes encoding phospho-lipolytic enzymes

To obtain all ten genes encoding phospho-lipolytic enzymes (Table 12) in different plasmids, a general cloning strategy was developed. Initially, two PCR reactions were performed per gene using two different primer pairs. PCR with the primer pair PAxxxxUp/PAxxxxDown (Table 6, where PAxxxx represents the PA number of the gene) led to a DNA fragment containing the complete gene sequence, whereas PCR with the PAxxxxUp/PAxxxxDownHis6 primer pair resulted in a gene encoding for a protein with a hexahistidine peptide directly after the carboxy terminal amino acid of the native protein. In each PCR product, *Nde*I and *Sac*I restriction sites were introduced in front of the start and behind the stop codon, respectively, to allow cloning into the pET22b(+) vector using *Nde*I and *Sac*I. In the subsequently obtained expression plasmids, genes were under the control of the T7-promoter (P_{T7}). Furthermore, each gene was subcloned from the pET22b(+) construct to the pBBR1mcs-3 plasmid using *Xba*I and *Sac*I restriction sites. The resulting plasmid carries the inserted gene together with the ribosome binding site of pET22b(+) downstream of the Lac promoter (P_{lac}). Additionally, each gene was subcloned from pET22b(+) into the pBR22b middle copy broad host range vector using *Nde*I and *Sac*I restriction sites, producing a T7-controlled expression vector suitable for expression in *P. aeruginosa* strains with the chromosomally encoded T7 RNA polymerase gene PAFR T7.7 or PABS T7.1 [161]. All constructed plasmids were validated in terms of gene, RBS and promoter accuracy by DNA sequencing.

4.3.2. Optimisation of heterologous protein expression and enzymatic activities of phospho-lipolytic enzymes

A set of experiments was performed to express each phospho-lipolytic enzyme with and without a His6-tag in homologous (*P. aeruginosa*) and heterologous (*E. coli*) hosts.The data for the expression of recombinant enzymes in *E. coli* strains will be presented in this chapter. For our approach, different overexpression methods were applied for expression in different bacterial strains using various expression plasmids (Chapter 4.3.1). Thus, P_{T7}-based overexpression in *E. coli* BL21(DE3) was induced with IPTG and "auto-induction" medium. P_{lac}-based expression was performed with or without IPTG induction in *E. coli* DH5α.

4.3.2.1. Expression and function of TesA

TesA was overexpressed in *E. coli* BL21(DE3) from the pET22b-TesA plasmid under standard IPTG inducing conditions (Chapter 3.1.1). Overexpression was monitored during the first three hours after induction and overnight by SDS PAGE analysis and activity assay. The culture samples at each time point were collected, and total cell lysates were prepared. SDS PAGE analysis revealed the appearance of an approximately 20 kDa protein in induced cells that was not observed in non-induced cells and cells transformed with empty vector (Figure 28A). This result is consistent with the predicted molecular weight of TesA, which is 21.0 kDa for the full-length protein and 18.9 kDa for the mature protein (without the putative signal sequence, Table 14). Therefore, the expressed 20 kDa protein is likely recombinant TesA. Separation of the inclusion bodies and soluble proteins of TesA expressing cells indicated the presence of TesA exclusively in the soluble form (data not shown). Therefore, we were encouraged to test whether TesA is a biologically active esterase as was predicted by the homology search (Chapter 4.2.2). Samples with confirmed overexpression of soluble TesA by SDS PAGE analysis were subjected to an esterase activity assay (pNPC substrate). The esterase activity of the induced samples was significantly higher than that of non-induced samples and empty vector samples (Figure 28B). The drastically decreased esterase activity obtained after overnight expression cannot be explained by degradation of TesA because the amount of TesA 3 h after induction and overnight are roughly the same according to the SDS PAGE gel (Figure 28A).

Taken together, the results show that TesA, a putative thioesterase of *P. aeruginosa* PA01, was successfully expressed as recombinant ca. 20 kDa protein that displayed esterase activity. TesA is the third esterase and second GDSL-hydrolase identified in *P. aeruginosa* PA01, after the previously described EstA (GDSL-hydrolase) and esterase encoded by orf. *PA3859* (Table 1). The TesA enzyme was examined in detail, and the results will be presented later, in Chapter 4.4.2.

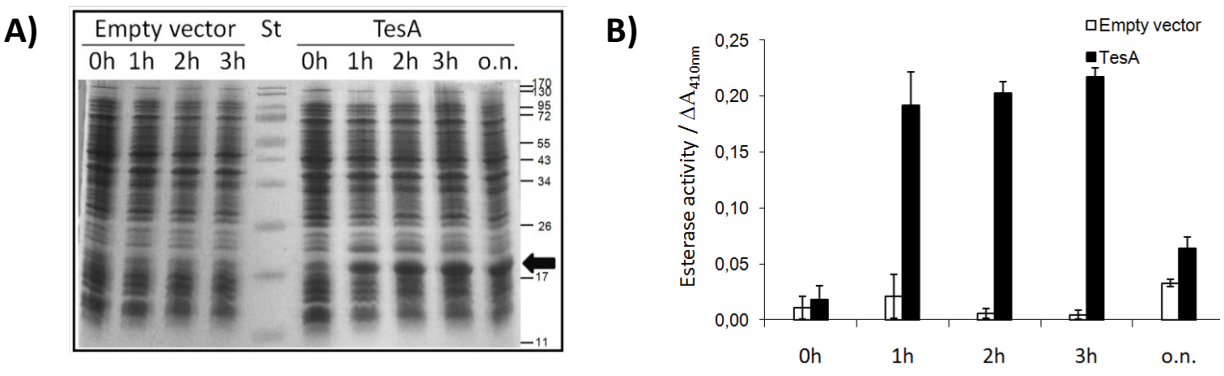


Figure 28: Overexpression and esterase activity of recombinant TesA. **A)** Coomassie-stained SDS PAGE gel (14%) of TesA overexpressed in *E. coli* BL21(DE3). The gel was loaded with 0.1 U_{Cell} of total cell lysates collected before induction (0h) and after 1 h, 2 h and 3 h as well as after overnight (o.n.) induction with IPTG. Molecular weights of protein standard (St) in kDa are indicated on the right. Empty vector = pET22b, TesA = pET22b-TesA. Black arrow indicates overexpressed TesA. **B)** The esterase activity of samples presented on SDS PAGE gel (Figure 28A). The esterase assay was performed with 10 µl of total cell lysate (0.01 U_{Cell}) per 1 ml of substrate solution, and the A_{410nm} was measured after 15 min. The results are the mean of three measurements, each performed in triplicate.

4.3.2.2. Expression and function of LipF

The expression of LipF was carried out in *E. coli* BL21(DE3) using the pET22b-LipF plasmid. Culture samples before induction and 1 h and 4 h after induction were collected, and total cell lysates were analysed by SDS PAGE. On the SDS PAGE gel, an additional protein band that migrated as an approximately 30 kDa protein was observed in samples after induction of overexpression, but not in negative controls (Figure 29A). The calculated molecular weight for full length LipF is 33.5 kDa, and the calculated molecular weight of the mature LipF without the first 20 aa, which are predicted to be a signal sequence (Table 14), is 31.5 kDa. According to the theoretical molecular weight of LipF, it is feasible that additional protein band observed in overexpression samples indeed represents LipF. To explore whether LipF was expressed as a soluble protein, separation of inclusion bodies and the soluble fraction was performed on the overexpression sample. SDS PAGE analysis revealed that the protein band that may be LipF is mainly present in the inclusion body fraction, but a weak LipF band was visible in the soluble fraction as well (Figure 29B). The question is whether the LipF band observed in the soluble fraction originated from soluble or membrane-bound LipF, as transmembrane helix for LipF was predicted by *in silico* methods (Table 15). However, in contrast to LipF present in inclusion bodies, soluble or membrane-associated LipF should be a catalytically active enzyme. To discriminate between these two possibilities, we decided to examine the catalytic activity of LipF.

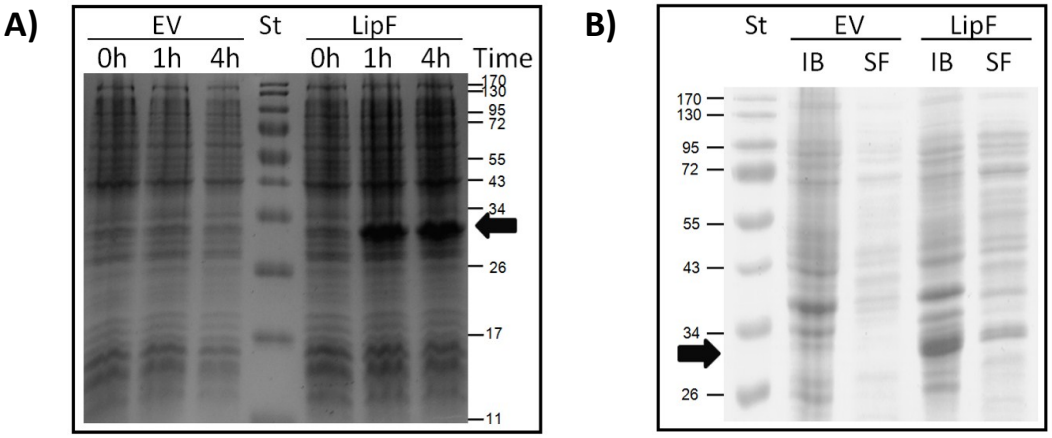


Figure 29: Overexpression of LipF. **A)** Coomassie-stained SDS PAGE gel (14%) of LipF overexpressed in *E. coli* BL21(DE3) under IPTG induction conditions. The gel was loaded with 0.1 U_{Cell} of total cell lysates collected before induction (0h) and 1 h and 4 h after induction with IPTG. **B)** Coomassie-stained SDS PAGE gel (14%) to analyse LipF solubility after overexpression in *E. coli* BL21(DE3). Molecular weights of protein standard (St) in kDa are indicated on the side. EV = pET22b, LipF = pET22b-LipF. IB = inclusion bodies, SF = soluble fraction. Black arrows indicate overexpressed LipF.

In Chapter 4.2.2, it was described that a bioinformatics search predicted lipase or esterase activity for LipF. Therefore, assays for lipase and esterase activity were used to test whether LipF is expressed as a native enzyme. The measurements in total cell lysates did not yield any increase of activity in samples containing overexpressed LipF compared to empty vector control samples (data not shown). These results strengthen our assumption that LipF is likely to be expressed as an inactive protein in the form of inclusion bodies. Known methods to reduce inclusion body formation are based on regulation of rate of protein synthesis with temperature [286] or partial induction [287]. Therefore, with the aim of maintaining LipF in a soluble form, overexpression in *E. coli* BL21(DE3) was performed at different cultivation temperatures (17˚C, 30˚C and 37˚C) and using different concentrations of IPTG inducer (0.1 mM and 0.4 mM final concentration). However, these approaches of modified expression of soluble LipF protein failed.

Next approach was to recovere LipF from inclusion bodies in its native conformation applying *in vitro* refolding. As it is advantageous to use a pure protein in refolding experiments, LipF tagged with a carboxy terminal His6-tag (LipFH6) was expressed (from pET22b-LipFH6 plasmid) under the same conditions as LipF (see above). SDS PAGE analysis of LipFH6 expression revealed an additional protein band that migrated at approximately 30 kDa in the samples after induction of overexpression, but not in non-induced samples (Figure 30A). Comparison of the size of LipFH6 and LipF on the SDS PAGE gel showed a slight increase of the Mw of LipFH6 compared to LipF as would be expected due to the fused His6-tag (Figure 30A). Further tests showed that LipFH6 had the same properties as LipF; it was not active in esterase or lipase assays, and it was present in inclusion bodies (data not shown). Therefore, inclusion bodies of LipFH6 expression were solubilised using urea and purified on Ni-NTA material under denaturation conditions (Figure 30B) (Chapter 3.16). Presence of an intense protein band corresponding to LipFH6 in the flow through (Figure 30B) indicated that LipFH6 was not fully bound to the material. The reason could be low affinity of LipFH6 to the Ni-NTA material because the same effect was observed in more experiments using different Ni-NTA chromatographic columns. Furthermore, the observation that a majority of bound LipFH6 was eluted from the column with a 50 mM concentration of imidazole indicates low affinity of LipFH6 for Ni-NTA material. The results revealed a smaller yield from purification but a satisfactory purity of LipFH6 to perform *in vitro* renaturation.

Prior the refolding experiments fractions of LipFH6 eluted from Ni-NTA columns with 50 mM and 250 mM imidazole were pooled, and the buffer was exchanged to Tris-HCl buffer (100 mM, pH = 8) supplemented with 8 M urea. The purity of the sample used in renaturation experiments is shown in Figure 30B (conc. LipFH6). A screening of six different renaturation buffers was performed by diluting the denatured LipFH6 sample 20-fold with renaturation buffers (Table 10) followed by incubation overnight at 4˚C. Due to the high dilution of LipFH6 during renaturation, the concentration of the protein sample used for renaturation needs to be sufficiently high, so we used sample with final LipFH6 concentration of 0.35 mg/ml. The efficiency of folding was monitored by esterase and lipase activity assays (Figure 31). We observed an increase in lipase activity (measured with pNPP) after renaturation of LipFH6 with all six buffers. However, two buffers (buffer 1 and 4), which recovered the highest activity of LipFH6, combine three different stabilizing reagents (Triton X-100, sucrose and arginine). For example, buffers containing Triton X-100 and sucrose (buffers 2 and 5) or buffers containing only Triton X-100 (buffers 3 and 6) are three-to-five-fold less efficient at renaturation compared to buffers containing Triton X-100, sucrose and arginine. Furthermore, we noted slightly

higher lipase activity of LipFH6 when comparing high ionic strength (buffers 4, 5 and 6) with low ionic strength (buffers 1, 2 and 3) renaturation buffers. The results of esterase activity assay with renatured LipFH6 (data not shown) were not interpretable due to high auto-hydrolysis of esterase substrate (pNPC) in renaturation buffers, which lead to measured artificial activity. However, it is common that enzymes that prefer long chain fatty acid esters (like it is pNPP) cleave short chain fatty acid esters (like it is pNPC) at a much lower rate. This was previously shown for LipC and LipA of *P. aeruginosa* [172].

We can conclude that LipF and LipFH6 were successfully expressed in *E. coli* BL21(DE3) as an approximately 30 kDa protein in the form of enzymatic inactive aggregates (inclusion bodies). Furthermore, LipFH6 was purified and renatured, yielding an enzymatically active protein that exhibited lipase activity. This enzyme is named lipase F (or LipF), and it is third lipase of *P. aeruginosa* besides the previously described LipA [24] and LipC [27] (Table 1).

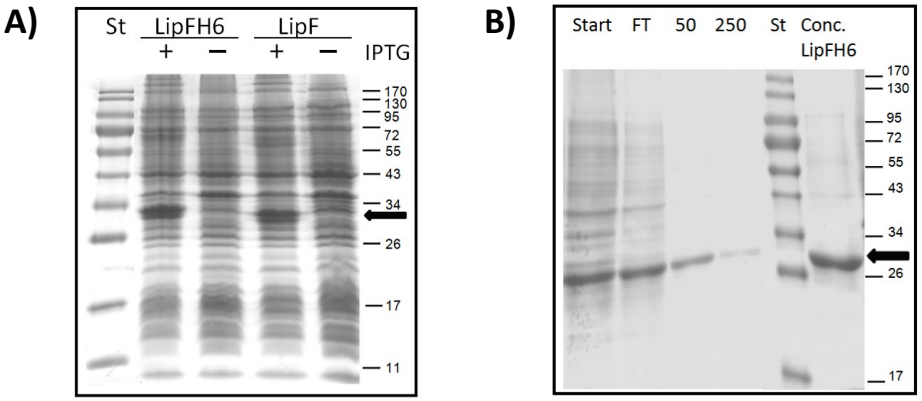


Figure 30: Expression and purification of LipFH6. **A)** Coomassie-stained SDS PAGE gel (14%) of LipF and LipFH6 overexpressed in *E. coli* BL21(DE3) under IPTG inducible conditions. **B)** Purification of LipFH6 on a Ni-NTA column. The gel was loaded with 0.1 U_{Cell} of total cell lysates collected before induction (0h) and 4 h after induction with IPTG. Black arrow indicates overexpressed LipF and LipFH6. Molecular weights of protein standard (St) in kDa are indicated on the right. LipF = pET22b-LipF, LipFH6 = pET22b-LipFH6, Start = solubilised LipFH6 inclusion bodies, FT = flow through, 50 and 250 = elution with 50 mM and 250 imidazole, respectively, Conc. LipFH6 = LipFH6 after concentration by ultrafiltration.

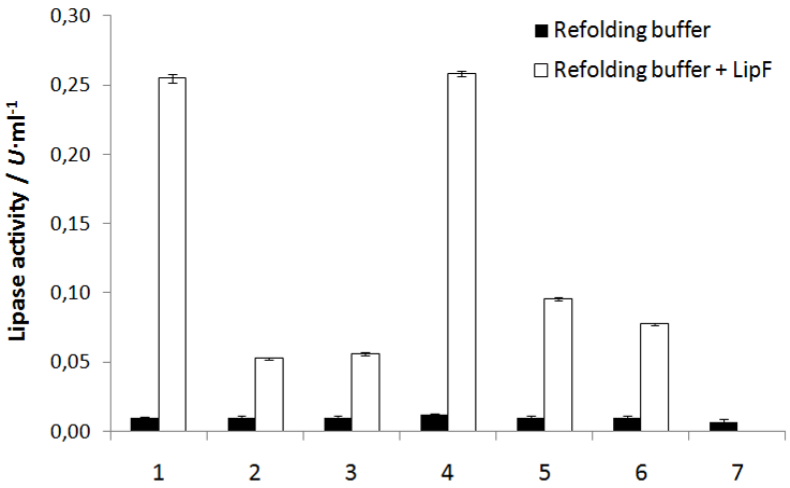


Figure 31: Refolding of LipFH6 monitored by lipase activity assay. The assay was performed with 10 µl (17.5 µg/ml) of renatured enzyme and renaturation buffer per 200 µl of substrate solution and the increase in A_{410nm} was continuously monitored over 10 min. 1 to 6 represent the six refolding buffers (see table 10, page 40) used in refolding the denatured inclusion bodies of LipFH6 (DIB). The results are the mean of two measurements, each performed in triplicate.

4.3.2.3. Expression and function of PlpD

Recombinant PlpD was expressed in *E. coli* BL21(DE3) from the pET22b-PlpD plasmid under IPTG inducing conditions and auto-induction conditions. Culture samples before IPTG induction and 2 h after induction were collected, and total cell lysates were analysed by SDS PAGE (Figure 32A). Additionally, a sample after 18h of auto-induction based overexpression of PlpD was compared to the empty vector sample (Figure 32A). From the SDS PAGE gels, it appeared that an additional protein with a size between 72 kDa and 94 kDa was present in PlpD overexpression samples, but not in empty vector controls. Prediction of PlpD function (Chapter 4.2.2) demonstrated a possible modular organization of PlpD that includes a membrane associated β-barrel domain and a catalytic patatin-like phospholipase domain and would result in a full-length PlpD with a molecular weight of 80.9 kDa. The theoretical molecular weight of PlpD fits to the observed results on the SDS PAGE gel. Therefore, it is likely that recombinant PlpD is expressed as a full-length protein. Moreover, the separation of inclusion bodies and the soluble fraction of overexpression samples revealed that the protein band probably represents PlpD is present in inclusion bodies and the soluble fraction (Figure 32B). SDS PAGE gel showed remarkably higher amount of recombinant PlpD in the inclusion body fraction however the portion of PlpD present in the soluble fraction could be the catalytically active protein. Therefore, lipase and esterase activity assays were carried out with the total cell lysates of PlpD-expressing cells (Figure 33). Although the esterase activity assay results were negative (data not shown), the lipase activity assay displayed lypolitic activity of PlpD. An increase of lipase activity was observed in samples after induction of PlpD expression in comparison to the empty vector samples and samples before induction.

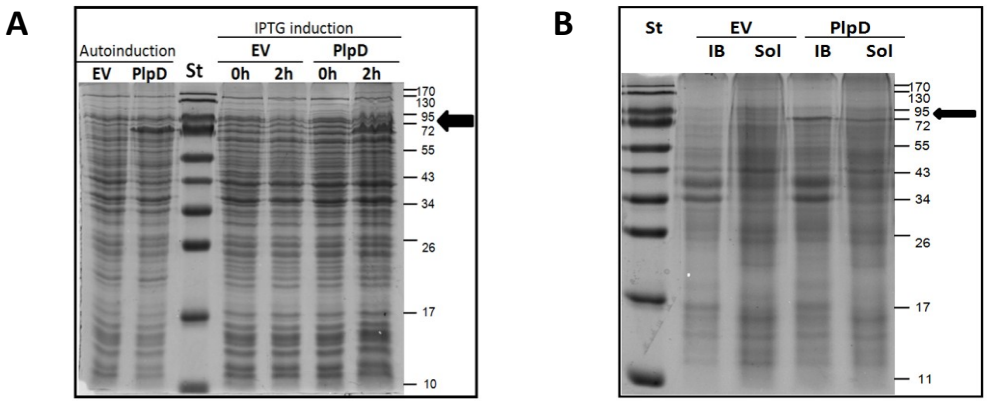


Figure 32: Coomassie-stained SDS PAGE gel (14%) of **A)** PlpD overexpressed under IPTG inducing and auto-induction conditions and **B)** solubility analysis of PlpD overexpression in *E. coli* BL21(DE3). Molecular weights of protein standard (St) in kDa are indicated on the right. PlpD = pET22b-PlpD, EV = pET22b. The gel was loaded with 0.1 U_{Cell} of total cell lysates collected before induction (0h) and 2 h after induction with IPTG as well as after auto-induction. Equivalents of 0.1 U_{Cell} of inclusion bodies (IB) and soluble fraction (SF) were analysed. Black arrow indicates overexpressed PlpD.

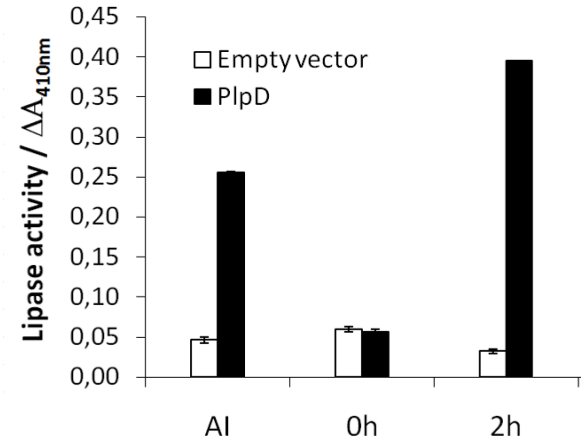


Figure 33: Lipase activity of recombinant PlpD expressed from the pET22b-PlpD plasmid in *E. coli* BL21(DE3) under auto-induction (AI) and IPTG induction conditions. 0h = before induction and 2 h = 2 h after induction. The assay was performed with 10 µl of total cell lysate (0.1 U_{Cell}) per 200 µl of substrate solution and the increase in A_{410nm} was continuously recorded over 20 min. The results are the mean of three measurements, each performed in triplicate.

We have observed that recombinant PlpD overexpressed under the control of a strong promoter, P_{T7}, is mostly found in inclusion bodies (Figure 32B), and it has been reported that the main reason for formation of inclusion bodies in *E. coli* is too high of a synthesis rate [286]. Furthermore, PlpD is predicted to be a membrane protein (Chapter 4.2.3), and in the literature, it was described that a high level of expression of membrane proteins may be lethal for *E. coli* [288] or can lead to insufficient translocation and insertion in membrane [289] leading to formation of inclusion bodies because membrane proteins displays hydrophobic nature and therefore aggregate in the aqueous environment. Therefore, we were decided to express PlpD under the control of uninduced P_{lac} in attempt to obtain low level expression resulting in higher amount of catalytically active PlpD. Thus, PlpD was expressed in *E. coli* DH5α from pBBR1mcs3-PlpD plasmid under the control of P_{lac} without induction. The empty vector and PlpD expression cultures were grown over night at 37˚C in LB medium and total cell lysates and supernatants were subjected to the lipase activity assay and SDS PAGE analysis. While expression of PlpD from uninduced P_{lac} was useful for activity assay (Figure 34), the amount of enzyme produced was not sufficient for efficient visualization by standard SDS PAGE analysis. Therefore, it seems that lower expression rate of PlpD under P_{lac} did not result in an

absolute increase of PlpD activity compared to the results obtained from PT7 dependent expression, but probably the ratio of active protein to produced protein is much better.

We can conclude that recombinant PlpD is expressed in *E. coli* using different expression conditions as a full-length ca. 80 kDa protein. This putative patatin like protein is characterised as a novel lipase of *P. aeruginosa* PA01 that belongs to the novel subfamily of autotransporter protein [247].

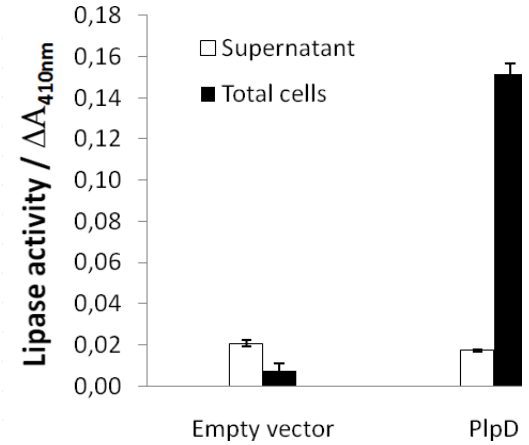


Figure 34: Lipase activity of PlpD expressed under the control of P_{lac} in *E. coli* DH5α from pBBR1mcs3-PlpD. The assay was performed with 10 µl of of supernatant (0.02 U_{Cell}) and total cell lysate (0.05 U_{Cell}) per 200 µl of substrate solution and increase of A_{410nm} was continuously recorded during 20 min. The results are mean of three measurements each performed in triplicates.

4.3.2.4. Expression and function of PlaK

PlaK was heterologous expressed in *E. coli* BL21(DE3) from pET22b-PlaK plasmid under the standard IPTG inducing conditions. SDS PAGE analysis of PlaK overexpression (Figure 35) indicates strong additional band which appears in sample after induction but is missing in empty vector sample and sample before the induction. The additional band in PlaK overexpression sample migrate as ca. 40 kDa protein what is in agreement with the theoretical molecular weight of 42.3 kDa calculated for PlaK. Furthermore the solubility analysis of PlaK overexpression sample reveals presence of putative PlaK in soluble fraction (data not shown). As the PlaK is putative esterase the esterase (pNPC substrate) and lipase (pNPP substrate) assays were applied to test whether PlaK is overexpressed as a active enzyme. The results indicate significant increase of esterase activity but not lipase activity of strain expressing PlaK in comparison to the empty vector control (Figure 35B).

SDS PAGE analysis and activity assay allow us to conclude that PlaK, a novel esterase of *P. aeruginosa* PA01 was successfully expressed as a recombinant ca. 40 kDa protein. Further details about this novel hydrolase of *P. aeruginosa* PA01 will be present in a chapter 4.4.1.

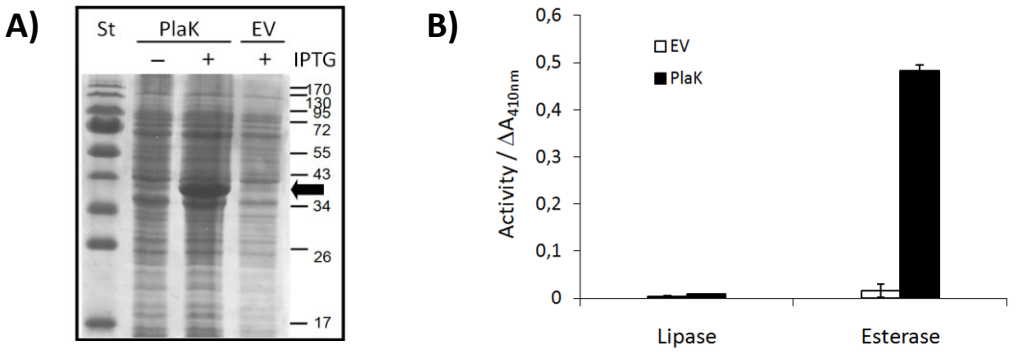


Figure 35: Overexpression of recombinant PlaK. **A)** Coomassie-stained SDS PAGE gel (14%) of PlaK overexpressed in *E. coli* BL21(DE3) from pET22b-PlaK (PlaK) compared to the empty vector control (EV, pET22b). The gel was loaded with 0.1 U_{Cell} of total cell lysates collected before induction (- IPTG) and 2 h after induction with IPTG (+ IPTG). Black arrow indicates overexpressed PlaK. Molecular weights of protein standard (St) in kDa are indicated on the right. **B)** Lipase (pNPP) and esterase (pNPC) activities of PlaK and negative control (EV) samples. The assay was performed with 10 µl of total cell lysate (0.15 U_{Cell}) per 200 µl of substrate solution and increase of A_{410nm} was continuously recorded during 10 min. The results are mean of two measurements each performed in triplicates.

4.3.2.5. Expression and function of PlaB

The heterologous overexpression of PlaB in a T7 RNA polymerase dependent system was tested with two plasmids in *E. coli* BL21(DE3). High-copy number pET22b-PlaB and medium copy number pBR22b-PlaB expression plasmids were used. Furthermore, the auto-induction and IPTG induction conditions were compared (Figure 36). The samples collected 4 h after IPTG induction (PlaBpET (+) and PlaBpBR (+)) reveal intensive protein band at the size between 43 kDa and 55 kDa. The same protein band appears in auto-induction samples but to a much higher yield than in sample after 4 h induction with IPTG. This band is not detectable in samples before induction (Figure 36) as well in the empty vector controls (data not shown) therefore it could represents recombinant PlaB. The molecular weight of full length PlaB is calculated to be 49.5 kDa therefore it is likely that observed protein band in overexpression samples is indeed PlaB. The solubility analysis of PlaB expressed under auto-induction conditions indicates possible presence of PlaB in the soluble fraction even if the band corresponding to PlaB is also visible in the insoluble fraction. However, it is to notice similar protein profile of soluble fraction and inclusion bodies therefore is questionable the efficiency of separation of inclusion bodies from soluble proteins.

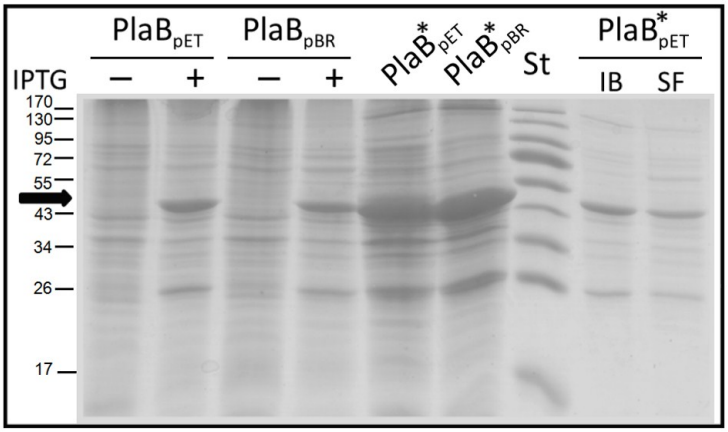


Figure 36: Coomassie-stained SDS PAGE gel (14%) of PlaB overexpressed in *E. coli* BL21(DE3) under the IPTG (+) induction and auto-induction conditions (denote by the star *). Molecular weights of protein standard (St) in kDa are indicated on the left. PlaB$_{pET}$ = pET22b-PlaB, PlaB$_{pBR}$ = pBR22b-PlaB. IB = inclusion bodies and SF = soluble fraction. The gel was loaded with 0.1 U$_{Cell}$ of total cell lysate collected before induction (0h), 2 h after induction with IPTG and after auto-induction. Black arrow indicates overexpressed PlaB.

To investigate if PlaB is overexpressed as native and active enzyme a set of activity assays; esterase (pNPC assay), lipase (pNPP assay) and phospholipase A2 were performed. The esterase and lipase activity measurements performed with total cell lysates did not turn out increased esterase or lipase activity in PlaB overexpression samples (data not shown). In the light of prediction of phospholipase A function of PlaB (Chapter 4.2.2) the PLA2 assay was performed with the PlaB overexpression samples. However, the results measured with total cell lysates (0.1 U$_{Cell}$/200 µl assay) were negative (data not shown). The localization prediction indicates possible membrane association of PlaB. For that reason the membrane fractions of cells overexpressing PlaB were isolated (Chapter 3.14.2) and respective PLA2 activities were measured (Figure 37). We have noticed that pET22b and pBR22b based expression systems reveals very similar expression yield of PlaB. In the PLA2 activity assay membrane fractions of pET22b-PlaB overexpression samples were used. We assumed that amount of correctly membrane inserted PlaB could be low and therefore large amount of membranes (equivalents to the 1 U$_{Cell}$) were used per activity assay (Figure 37). The results clearly indicate an increase of PLA2 activity measured with the PlaB overexpression samples (IPTG induction and auto-induction) in comparison to the empty vector controls and samples before induction of expression with IPTG. The activity of PlaB expressed under IPTG inducing conditions is lower than that expressed under the auto-induction conditions what could be explained by significantly higher PlaB amount after the auto-induction expression what is visible on the SDS PAGE gel (Figure 37).

We can conclude that overexpression of recombinant PlaB was successful but no esterase, lipase or PLA2 activities were measurable in total cell lysates. However, the membrane fraction of PlaB expressing strain reveals significant PLA2 activity which may be attributed to the membrane bound PlaB. This data of heterologous expression are indications that PlaB represents the first, probably membrane bound, PLA2 of *P. aeruginosa* PA01.

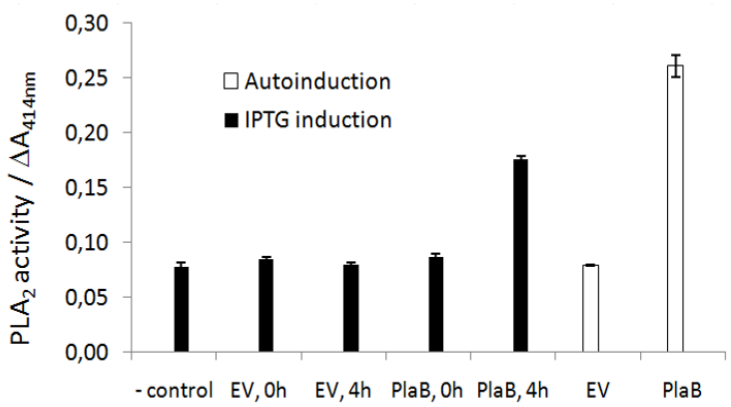


Figure 37: Phospholipase A2 activity of the membrane fraction of recombinant PlaB expressed in *E. coli* BL21(DE3) under IPTG induction and auto-induction conditions. The assay was performed with 10 µl of membrane fraction (1 U_{Cell}) per 200 µl of substrate solution and increase of A_{414nm} was monitored during 30 min. Like an negative control (- control) was used sole buffer used for membrane fraction resuspension (Tris-HCl, 100 mM, pH = 8), EV = empty vector (pET22b) and PlaB = pET22b-PlaB plasmid, with 0h and 4 h mean the sample before induction and 4 h after induction with IPTG.

4.3.2.6. Expression and function of PldC and EstC

The overexpression of PldC and EstC in heterologous host (*E. coli* BL21(DE3)) under the control of PT7 was tested using the pET22b-PldC and pET22b-EstC plasmids. The total cell lysates of overexpressions were analysed by means of SDS PAGE and activity assays. The SDS PAGE gel with overexpressed PldC reveals additional protein of ca. 40 kDa in samples after induction with IPTG in comparison to the sample before induction and empty vector control (Figure 38). The observation is in keeping with the theoretical molecular weight of full length PldC of 41.4 kDa. The EstC overexpression (Figure 39) shows an additional band in samples after the induction of expression with IPTG in comparison to non induced sample and empty vector control. The overexpressed protein falls in the range between 26 kDa and 34 kDa what corresponds to the theoretical molecular weight of EstC of 31.3 kDa. The SDS PAGE analysis of overexpressed PldC and EstC samples indicate successful overexpression of both proteins. Therefore those samples were subjected to the activity assays in order to find out whether PldC and EstC are expressed as native and active proteins. Phospholipase D and esterase activity was previously predicted for PldC and EstC respectively (Chapter 4.2.2). Therfore esterase and lipase activities of empty vector samples were compared with PldC and EstC overexpression samples. However no significant differences were obtained. The lack of activity assay specific for PLD disallows us to test whether PldC indeed possesses PLD activity. In light of these results it is not possible to conclude whether the EstC and PldC are expressed as non functional enzymes or the used substrates were not suitable to measure an activity of those two enzymes.

We can conclude that overexpression of PldC and EstC as recombinant proteins of ca. 40 kDa and ca. 30 kDa respectively was confirmed by the SDS PAGE analysis. Since both enzymes are not active as a lipase or an esterase, and further activity assays have to be performed to examine their exact enzymatic functions. So it is not possible to draw the conclusion whether they are functionally expressed and what are their actual enzymatic functions.

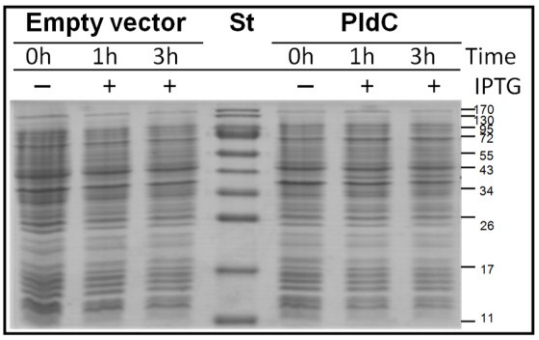


Figure 38: Coomassie-stained SDS PAGE gel (14%) of PldC overexpressed in *E. coli* BL21(DE3). Molecular weights of protein standard (St) in kDa are indicated on the right. Empty vector = pET22b, PldC = pET22b-PldC. The gel was loaded with 0.1 U_{Cell} of total cell lysate collected before induction (0h) and 1 h or 3 h after induction with IPTG. Black arrow indicates overexpressed PldC.

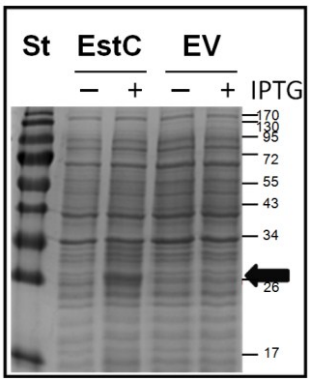


Figure 39: Coomassie-stained SDS PAGE gel (14%) of EstC overexpressed in *E. coli* BL21(DE3). Molecular weights of protein standard (St) in kDa are indicated on the right. EV = pET22b, EstC = pET22b-EstC. The gel was loaded with 0.1 U_{Cell} of total cell lysates collected before induction (0h) and 2 h after induction with IPTG. Black arrow indicates overexpressed EstC.

4.3.2.7. Expression and function of EstB, PldB and PlbF

The last three phospho-lipolytic enzymes (EstB, PldB and PlbF) were overexpressed in *E. coli* BL21(DE3) under the standard overexpression conditions from respective pET22b and pBR22b based plasmids. The total cell lysates of overexpression samples were analyzed by means of SDS PAGE but no indications about the overexpression of any of those three proteins were observed (data not shown). Very high expression level of recombinant proteins was reported to have many negative effects on the *E. coli* cells what invokes variety of cellular stress responses. One of this responses is an increased proteolysis responsible for degradation of recombinant protein [286]. A decreased synthesis rate of recombinant protein can be energetically beneficial to the cells [290], thus reducing proteolysis what consequently may yield higher amount of recombinant proteins. Since proteolysis can be reason of lack of overexpression bands which could be attributed to the EstB, PldB and PlbF after the strong P_{T7} controlled overexpression we have decided to use weaker P_{lac} in order to decrease expression rate. By this approach reduced expression level may cause less stress for the cells and result with a sufficient amount of protein for efficient visualization by standard SDS PAGE analysis. Therefore, the EstB, PldB and PlbF were expressed in *E. coli* DH5α from respective pBBR1mcs-3 plasmids under the control of P_{lac}. Unfortunately, the SDS PAGE analyses do not reveal positive overexpression for those enzymes (data not shown).

Although the overexpression of EstB, PldB and PlbF were not detectable by SDS PAGE analysis the esterase and lipase activity assays were routinely performed with total cell lysate of *E. coli* BL21(DE3) overexpression samples. Although lipase activity assay (pNPP) have not shown positive result for the PlbF overexpression samples (*E. coli* BL21(DE3) and *E. coli* DH5α) they were positive in esterase activity assay (pNPC) (Figure 40).

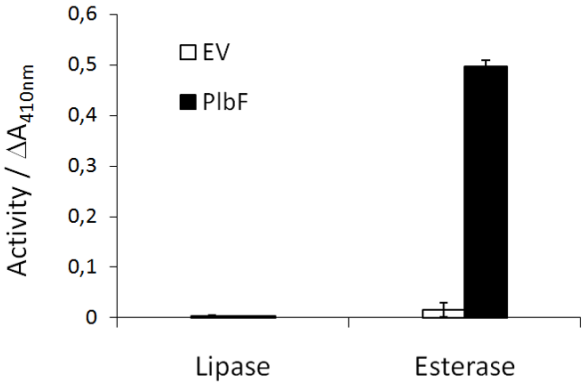


Figure 40: Lipase and esterase activities of the total cell lysate of recombinant PlbF expressed in *E. coli* BL21(DE3) under autoinduction conditions. The assay was performed with 10 µl of total cell lysate (0.15 U_{Cell}) per 200 µl of substrate solution and increase of A_{410nm} was continuously recorded during 10 min. The results are mean of two measurements each performed in triplicates. EV = empty vector (pET22b), PlbF = pET22b-PlbF expression plasmid.

We can conclude that EstB and PldB are either not expressed in *E. coli* or they are expressed on low level but are not active with tested lipase and esterase substrates. Even, though PlbF is not expressed in *E. coli* in sufficient amount to be detected on SDS PAGE gel but it was shown that PlbF exhibits esterase activity. PlbF is one of three members of phospho-lipolytic system (togetger with PlaK and TesA) which will be later presented separately (chapter 4.4.3).

Table 16: Expression and enzymatic activities of ten novel hydrolases from *P. aeruginosa* PA01 produced in the heterologous host *E. coli*. SDS PAGE = protein is detected by visualisation on standard SDS PAGE gel; PLA2 = phospholipase A2 activity (Chapter 3.11.4); ND = not determined.

Enzyme Name	SDS PAGE	Activity assay		
		Esterase	Lipase	Other
PlaK	+	+	-	ND
EstB	-	-	-	ND
PldB	-	-	-	ND
TesA	+	+	-	ND
PlaB	+	-	-	+ PLA2
PlbF	-	+	-	ND
PlpD	+	-	+	- PLA2
EstC	+	-	-	ND
PldC	+	-	-	ND
LipF	+	-	+	ND

Summary

4.3 Molecular cloning, expression, purification and biochemical characterisation of phospho-lipolytic enzymes

Data obtained by systematic expressionng and examination of the enzymatic functions of ten novel putative hydrolases of *P. aeruginosa* PA01 (phospho-lipolytic enzymes) are presented in Table 16. These data show that eight of ten enzymes were indeed detected as recombinant proteins in *E. coli*. Furthermore, seven of them were expressed in sufficient amount to be visualised by standard SDS PAGE method. Although PlbF was not detected by means of SDS PAGE, its expression was sufficient for demonstration of its esterase activity in total cell lysates. Beside PlbF, another two enzymes (PlaK and TesA) were characterised as esterases. For two enzymes (LipF and PlpD) lipase activity was demonstrated and one enzyme (PlaB) was characterised as a

4.4. Biochemical characterisation and localisation of novel phospholipases

Our previous results demonstrated that *P. aeruginosa* PA01 expresses PLA2 and LysoPLA activities (Chapter 4.1). Furthermore, some candidates that may possess PLA2 and LysoPLA activities (named "phospho-lipolytic enzymes") have been identified using bioinformatics (Chapter 4.2). Finally, heterologous expression of ten phospho-lipolytic enzymes revealed the expression conditions and enzymatic functions for eight candidates (Chapter 4.3). The next step was to choose the most promising candidates and explore them in greater detail, in terms of their physicochemical properties and roles in cell pathophysiology. The criteria for choosing interesting candidates were as follows:

A) Novel features recognized from primary protein structures

B) Feasibility of homologous and heterologous protein expression and detection

C) Novel activities (Phospholipase A/B or Lysophospholipase) previously undescribed in *P. aeruginosa* PA01

D) Putative extracellular localization because secreted phospholipases have strong virulence factor potential

We have focussed on three putative phospholipases TesA, PlbF and PlaK. For first two proteins were predicted signal peptides suggesting putative extracellular localisation. The third one is a putative esterase with an active site motif characteristic of the β-lactamase family suggesting its extracytoplasmic localisation.

4.4.1. PlaK, a multifunctional enzyme possessing esterase, β-lactamase and phospholipase A activities

Although PlaK was not predicted to be a PLA or LysoPLA, neither was it predicted to be an extracellular protein, we have decided to explore this particular phospho-lipolytic enzyme in greater detail. The main reason is the prediction that PlaK is an esterase and β-lactamase without a typical esterase sequence motif (GXSXG) than serine β-lactamase motif (SXXK) that was recognized in PlaK. In the literature, an example of such an enzyme has been described, EstB from *B. gladioli* [205], with a SXXK (serine β-lactamase) motif and esterase but not β-lactamase activity. This phenomenon, namely a preference for esterase substrates over β-lactamase substrates, has been elucidated on the structural level, because the crystal structure of EstB from *B. gladioli* is available. However it is tempting to speculate that PlaK might be the first described esterase that possesses at the same time β-lacatamase activity.

Furthermore, the predicted β-lactamase activity of PlaK was an additional motive to explore this enzyme. *P. aeruginosa* displays inherent resistance to many drug classes (multiple resistance) [291] but especially problematic is its resistance to the most widely-used antibiotics belonging to the β-lactam family. The important mechanism for inactivation of β-lactam antibiotics is production of various β-lactam hydrolysing enzymes called β-lactamases [292]. Therefore, any knowledge about novel β-lactamases in *P. aeruginosa* PA01 may contribute to a better understanding of antibiotic resistance in this species.

Our aim was to elucidate if PlaK is an esterase and/or β-lactamase using the pure PlaK enzyme. Furthermore, the potential of PlaK to hydrolyse PLA1, PLA2 and thioesterase substrates will be tested in order to explore the hydrolytic spectrum of this novel hydrolase. Homologous expression of PlaK will be performed in order to reveal its cellular localization.

4.4.1.1. Purification of recombinant PlaK

Our previous results showed overexpression of PlaK in high amounts in *E. coli* BL21(DE3) from a plasmid where PlaK is under the control of a P_{T7} (Figure 35). For purification purposes, we have tested the expression of PlaK tagged with a His6-tag at its carboxy-terminal end, using the pET22b-PlaKH6 plasmid under identical conditions to those that have been applied for expression of untagged PlaK (Chapter 4.3.2). The overexpression of PlaKH6 was comparable to the overexpression of untagged PlaK in terms of esterase activity and protein solubility (data not shown). We have detected catalytically active (with an esterase substrate) PlaKH6 in the soluble fraction, from which it was purified by means of Ni-NTA chromatography.

PlaKH6 bound to a Ni-NTA column was eluted in a stepwise manner with buffers contain 20 mM, 50 mM, 100 mM and 250 mM imidazole (Chapter 3.16). The PlaKH6 purification was followed by an esterase activity assay and SDS PAGE analysis. Purification analysis by means of SDS PAGE (Figure 41A) indicates that PlaKH6 had bound to the Ni-NTA material, because a decrease in the intensity of the PlaK overexpression band in the flow through compared to the soluble cell extract was observed. Furthermore, the presence of a ca. 40 kDa protein band was noticed, which may represent PlaK in fractions eluted with buffers containing 100 mM and 250 mM imidazole. Because the protein profiles

of those two fractions were the same, they were pooled and concentrated (Figure 41B). However, SDS PAGE reveals three pairs of protein bands: at ca. 40 kDa, ca. 90 kDa and ca. 130 kDa (Figure 41A). In the concentrated PlaKH6 sample (Figure 41B) at the size of 40 kDa, only one protein band is visible, which may be due to two overlapping bands. Our experience with purification of other His6-tagged proteins (from the same host and under similar conditions) by Ni-NTA columns did not show the presence of so many impurities (unwanted proteins) in the 100 mM and 250 mM fractions. Furthermore, it seems that these proteins bind to the Ni-NTA resin with an affinity comparable to PlaKH6. Therefore we have tested whether the observed higher molecular weight protein bands might be multimers of PlaKH6. Because PlaKH6 is tagged with the His6-tag we have attempted immunodetection of PlaK with antibodies against the His_6 peptide. The concentrated PlaKH6 sample was subjected to Western blotting (Figure 41C), and a strong signal at 40 kDa was detected. Furthermore, weak signals at ca. 90 kDa and 130 kDa, which may represent dimeric and trimeric form of PlakH6, were observed. Surprisingly the presence of PlaKH6 multimers even under the denaturing conditions used for protein separation on an SDS PAGE gel was detectable what should be further examined.

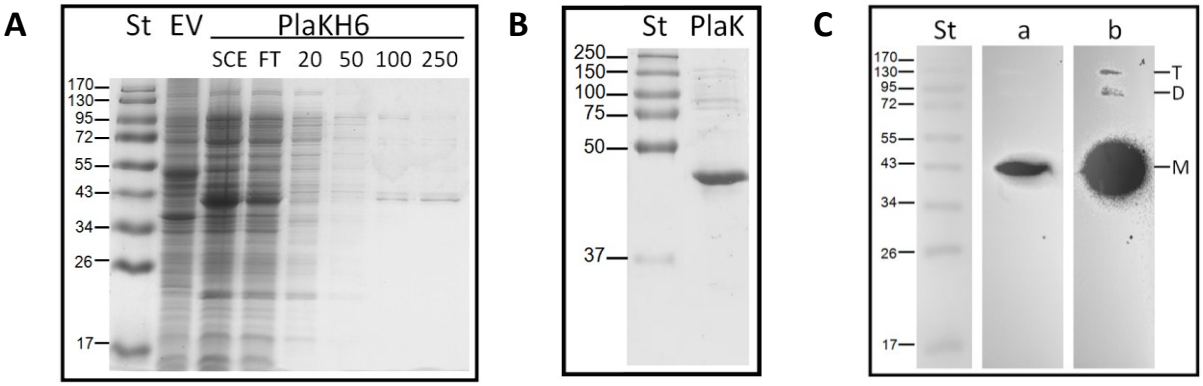


Figure 41: Purification and immunodetection of recombinant PlaKH6. **A)** Coomassie-stained SDS PAGE gel (14%) of PlaKH6 purification via a Ni-NTA gravity flow column (2 ml Ni-NTA resines) (Chapter 46). EV = soluble cell extract of empty vector (pET22b) cells; SCL = soluble cell extract of PlaKH6-expressing cells; FT = flow through; 20, 50, 100 and 250 = fractions eluted with Ni-NTA purification buffers containing 20, 50, 100 and 250 mM imidazole, respectively. **B)** Coomassie-stained SDS PAGE gel (14%) of concentrated PlaKH6 fractions 100 and 250 from Figure 41A. **C)** Western blot of concentrated PlaKH6 sample detected with anti-His6-tag HRP antibodies (Chapter 3.18.4). Lanes a and b represent visualization of the same Western blot membrane section after short and long time exposures, respectively. Signals of monomeric, dimeric and trimeric PlaKH6 are marked with M, D and T, respectively. Molecular weights of protein standard (St) in kDa are indicated on the right.

Concentrated PlaKH6 sample after exchanging its buffer against Tris-HCl buffer (100 mM, pH = 8) yielded more than 80% of the soluble cell extract activity for PlaKH6 (sample loaded onto the column). The loss of ca. 20% of enzyme activity is due to insufficient binding of PlaKH6 to the column, which was also observed by an SDS PAGE gel. The reason may be overloading of the Ni-NTA column, because only 2 ml of Ni-NTA material was used for purification of PlaKH6 from a 1 l culture. The purified PlaKH6, stored for long period at -20˚C in buffer supplemented with 20% (v/v) glycerol as a cryoprotectant, maintains full esterase activity.

4.4.1.2. PlaK is a β-lactamase and a phospholipase A2

Further examination of the enzymatic function of PlaK was performed with purified PlaK enzyme. It was previously mentioned that PlaK is predicted to be an esterase with the β-lactamase active site motif (SXXK). We have indeed demonstrated enzymatic activity of PlaK against an esterase substrate (pNPC). In the closing stage of our definition of the hydrolytic activity of PlaK, its enzymatic activity against the general β-lactamase substrate CENTA (Chapter 3.17.7) and a PLA2 substrate (Chapter 3.11.4) was explored. CENTA is a convenient chromogenic substrate that is used in the study of all classes of β-lactamase [171, 293]. The inhibition of PlaK esterase activity was readily accomplished by a short incubation of pure PlaKH6 with serine hydrolases inhibitor PMSF (100-fold molar excess, data not shown). This knowledge was used to prepare an inactive PlaKH6 enzyme that served as a negative control in the β-lacatamase and PLA2 assays. The esterase activity measured for PlaK and inactive PlaK has confirmed the enzymatic functionality of PlaK but not of inactive PlaK, as was expected (data not shown). Furthermore, the total cell lysate from *P. aeruginosa* PA01 was used as a positive control in the β-lacatamase assay because it has been described that *P. aeruginosa* generally expresses more intracellular β-lactamases [294]. The β-lactamase and PLA2 activity assays revealed that PlaK is indeed able to hydrolyse both substrates (Figure 42) but with different hydrolysis rates. The β-lactamase activity (43.7 U/μl) of PlaK is five-fold higher than its PLA2 activity (9 U/μl). However, the esterase activity (measured with pNPC) of the same PlaK sample used in the PLA2 and β-lactamase assays is 176.6 U/μl, underscoring its dominant function as an esterase. It should be noted that inactive PlaKH6 (negative control) does not display activity in either assay (Figure 42). Furthermore, positive control reactions demonstrate proper functioning of both assays.

At this point, we can conclude that PlaK is a *bona fide* esterase with a pronounced β-lactamase activity and additional PLA2 activity. However, this multifunctionality of PlaK indicates that this enzyme is a unique hydrolase, because to date an enzyme has not been described that possesses esterase, PLA2 and β-lactamase activities concomitantly.

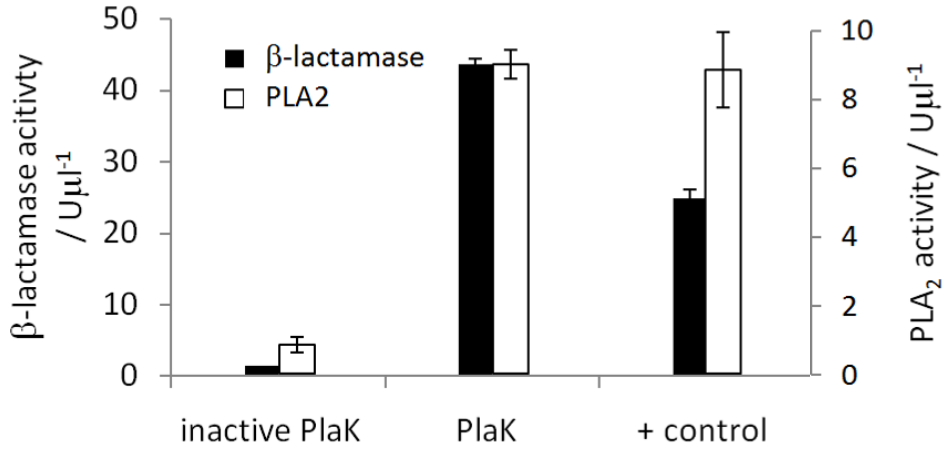


Figure 42: β-lactamase and PLA2 activities of PlaK. The β-lactamase activity assay was performed by incubating 10 μl sample (0.06 mg/ml PlaKH6) with 150 μl substrate at 30°C. The increase in A_{405nm} was continuously recorded during 30 min. The PLA2 activity assay was performed by incubating 10 μl sample (0.06 mg/ml PlaKH6) with 150 μl substrate and 10 μl DTNB at 30°C. The A_{414nm} was measured 17h after the reaction was initiated. As a positive control for the β-lactamase and PLA2 assays, 0.1 U_{cell} of *P. aeruginosa* PA01 total cell lysate and 10 μl bee venom PLA2 (1 ng/ml) were used, respectively. In both assays spontaneous hydrolysis of the substrate was measured appropriate

buffer and these values were subtracted from all measurements. The results are the mean of three measurements.

4.4.1.3. Expression and cellular localisation of PlaK in *P. aeruginosa* PA01

The expression of PlaK in *P. aeruginosa* PA01 should elucidate whether PlaK exhibits esterase activity and in which cellular compartment it is localised. An *in silico* prediction of the cellular localization for PlaK indicates a cytoplasmic localization, due to the lack of a putative signal peptide and membrane helix in its sequence (chapter 4.2). PlaK was expressed in the *P. aeruginosa* PA01 strain from a pBBR-PlaK plasmid, where it is controlled by P_{lac}. Expression was carried out overnight in rich medium (LB). The supernatant and total cell lysate from the overnight culture were subjected to the esterase activity assay (Figure 43). An increase in esterase activity for the strain expressing PlaK (defined as 100%), as compared to the empty vector strain (47% of activity of PlaK-expressing strain), was measured with the total cell lysate. The esterase activities of supernatants from both strains were low and roughly equal. Therefore, we conclude that PlaK is expressed in *P. aeruginosa* PA01 as a catalytically functional cell-associated esterase.

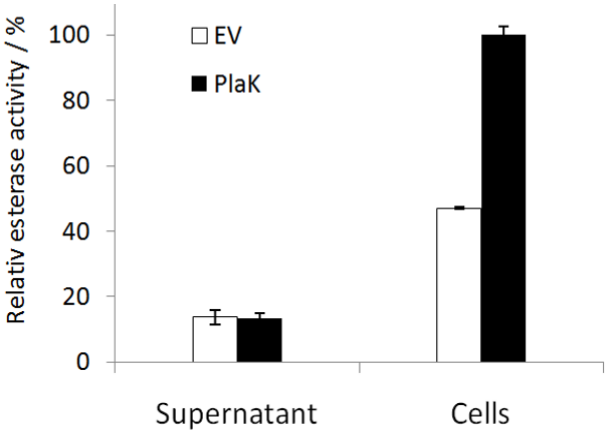


Figure 43: Esterase activity of *P. aeruginosa* PA01 expressing PlaK. The esterase activity assay (pNPC) was performed with 20 µl (equivalent to 0.1 U_{Cell}) supernatant and total cell lysate (cells) per 200 µl substrate solution, and the increase in A_{410nm} was continuously recorded during 15 min. Esterase activities are normalized to the activity of total cell lysate from *P. aeruginosa* PA01 carrying pBBR-PlaK (PlaK), which was defined as 100%. EV = *P. aeruginosa* PA01 carrying pBBR1mcs-3 (empty vector). The results are means of two measurements, each performed in triplicate.

In order to reveal whether PlaK is expressed as a soluble protein, as suggested by localisation predictions, immunodetection of PlaKH6 was used. PlaKH6 expressed in *P. aeruginosa* PA01 displays similar esterase activity to an untagged protein (data not shown). Therefore PlaKH6 was expressed in *P. aeruginosa* PA01 under the same conditions as above for PlaK and supernatant, membranes and soluble proteins were separated. Following growth of the *P. aeruginosa* PA01 strain carrying empty vector and PlaK expression vector, no significant differences were observed (Figure 44). Furthermore, the middle logarithmic and early stationary phase cultures were analysed, but differences in PlaK localization were not observed; therefore, only results with the middle logarithmic cultures are presented. Immunodetection of PlaKH6 with an anti-His6-tag antibody (Chapter 3.18.4) revealed the presence of PlaK only in the soluble fraction from a strain expressing PlaK (Figure 44). Because the soluble fraction is composed of periplasmic and cytoplasmic fractions, it is not possible to distinguish whether PlaK is a cytoplasmic or periplasmic protein. However, the immunodetection of DsbA, a periplasm-associated protein from *P. aeruginosa* PA01 [179], showed its presence in both the soluble fraction and the supernatant (Figure 44). This demonstrates leakage of periplasm in the supernatant upon expression of PlaK; therefore, periplasmic protein is detected in the culture supernatant. This

finding, moreover, has implications for the localization of PlaK, because if PlaK were a periplasmic protein it should be released together with DsbA into the culture supernatant. Consequently, the absence of PlaK in the culture supernatant indirectly indicates a cytoplasmic localization for PlaK.

Taken together, we can conclude that expression of PlaK in *P. aeruginosa* PA01 resulted in the presence of functional esterase in the soluble fraction (periplasm and cytoplasm together). Moreover, it was indirectly demonstrated that PlaK is a cytoplasmic but not periplasmic protein.

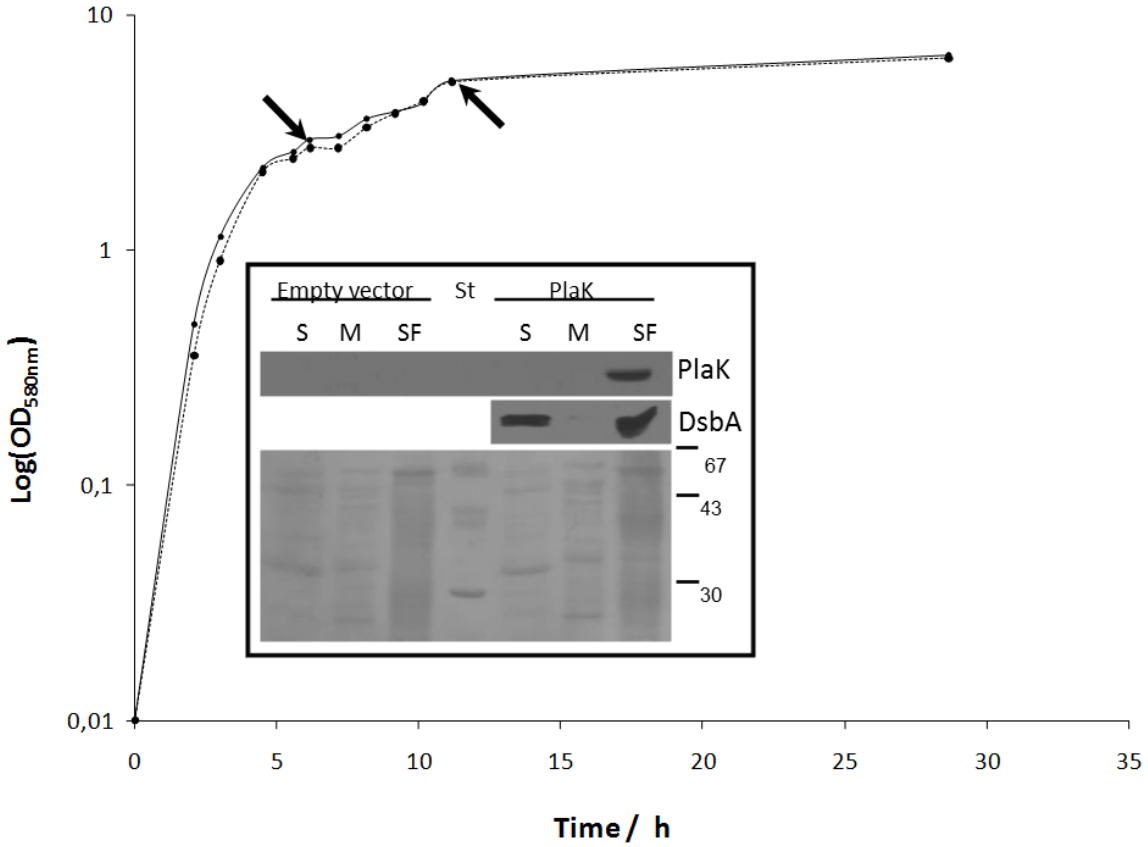


Figure 44: Subcellular localization of PlaK in *P. aeruginosa* PA01. Growth of *P. aeruginosa* PA01 transformed with pBBR1mcs-3 (empty vector, dashed line) and pBBR-PlaKH6 (PlaK, solid line) was compared under standard conditions. Surrounded by the black frame in the middle of the figure are Western blots (two upper panels) with detection of PlaKH6 and DsbA using anti-His6-tag HRP antibodies (PlaK) and anti-DsbA antibodies, respectively. The lower panel is the coresponding Coomassie-stained SDS PAGE gel (14%). S = supernatant, M = membrane, SF = soluble fraction. Molecular weights of protein standard (St) in kDa are indicated on the right. Cultures were examined at two growth points (indicated by black arrows) and identical results were obtained in both analyses, therefore only results with cultures from the earlier growth point are presented.

Summary

4.4.1 PlaK, a multifunctional enzyme with esterase, β-lactamase and phospholipase A activities

PlaK displays sequence homology to the group of β-lactamases and esterases whose putative active site contains a S_{78}XXK β-lactamase motif and Tyr167 (Figure 16). *In silico* prediction of PlaK cellular localization indicates a cytoplasmic localization due to lack of a putative signal peptide or membrane helix in its sequence (Chapter 4.2).

Heterologous expression of recombinant PlaK in *E. coli* BL21(DE3) was successful, resulting in detection of a ca. 40 kDa PlaK protein with pronounced esterase activity (Figure 35). Recombinant PlaK tagged with a carboxy-terminal His6-tag was purified on a Ni-NTA column. The analysis of PlaK purification by Western blot indicates existence of PlaKH6 multimers that are stable under the denaturing conditions applied for protein separation on an SDS PAGE gel.

Purified PlaK displays enzymatic activity against general β-lactamase (43.7 U/μl) and PLA2 (9.0 U/μl) substrates. Although these activities are lower than the esterase activity of PlaK (176.6 U/μl), we can conclude that PlaK is a novel esterase/β-lactamase/PLA2 from *P. aeruginosa* PA01. Such multifunctionality, namely an esterase with β-lactamase and PLA2 functions, has not been previously described in the literature and indicates that this enzyme is unique among bacterial hydrolases.

PlaK was expressed as a soluble (periplasmic or cytoplasmic) protein of *P. aeruginosa* PA01 that exhibits esterase activity. Moreover, indirect evidence reveals a cytoplasmic but not periplasmic localization for PlaK.

4.4.2. TesA, a periplasmic GDSL-hydrolase from *P. aeruginosa* with esterase and lysophospholipase A activities

TesA has received considerable attention due to its putative extracytoplasmic localization, putative PLA2 and LysoPLA activities and membership in the GDSL-hydrolase family, which is interesting from structural, physiological and evolutionary standpoints. A signal peptide is predicted for TesA, therefore one might expect that TesA is translocated trough the inner membrane into the periplasm or perhaps even to the extracellular space. Furthermore, TesA exhibits sequence homology to LysoPLA and PLA2. Extracellular hydrolases are attractive due to their possibility for interacting with host cells and in such a manner contributing to the virulence of the pathogen. Therefore, it is tempting to analyse whether TesA is a secreted enzyme with LysoPLA or PLA2 activity, which may suggest it is a potential virulence factor of *P. aeruginosa* PA01. TesA is structurally interesting because it is classified in the GDSL-hydrolase family, which is characterised by an α/β/α-fold that is distinct from the common α/β-hydrolase fold present in most if not in all hydrolytic enzymes. In the literature, GDSL-hydrolases have been described from diverse species, indicating their broad phylogenetic distribution and conservation over more than 200 million years [295], which may be related to the general importance of these enzymes. To date, only one member of this family has been described in *P. aeruginosa* PA01, namely esterase A (EstA). Although it is mentioned in the

Introduction section (Chapter 1.3), it is worthwhile to restate the role of EstA in rhamnolipid synthesis, biofilm production and the motility of *P. aeruginosa* PA01, phenotypes typically related to the pathogenicity [1]. Altogether, we were intrigued to explore the biological function of TesA, a novel hydrolytic enzyme of *P. aeruginosa* PA01.

Our aim was to purify TesA in order to define this novel hydrolase in terms of enzymatic activity. Therefore the PLA2, PLA1, thioesterase, esterase, lipase and protease activities of the pure enzyme were evaluated. The bioinformatic prediction of the TesA active site will be experimentally tested by site-directed mutagenesis of putative catalytic triad residues. Furthermore, basic physicochemical properties of TesA will be examined. Purified TesA will be subject to structure characterisation in order to understand the functional features of this enzyme. To discern the physiological role of TesA, the enzyme will be localized in *P. aeruginosa* PA01, and a strain lacking the *tesA* gene should be generated.

4.4.2.1. Expression and purification of TesA

Previously, we have demonstrated (Figures 28) successful heterologous overexpression of the TesA protein, which exibits enzymatic activity against an esterase substrate (pNPC). In order to purify TesA on a Ni-NTA column, the enzyme tagged with a hexahistidine peptide at its carboxy-terminal end (TesAH6) was expressed from the pET22b-TesAH6 plasmid in *E. coli* BL21(DE3) under the conditions identical to those used for expression of untagged TesA (Chapter 4.3.2). The comparison of tagged and untagged TesA in terms of expression, protein solubility and esterase activity (measured with total cell lysate) did not reveal significant differences (data not shown). Being convinced that TesAH6 is expressed as a soluble and enzymatically active protein, we purified it from a soluble cell extract via Ni-NTA chromatography. The stepwise elution of TesAH6 bound to a Ni-NTA column with buffers containing 20 mM, 50 mM and 250 mM imidazole reveals a pure ca. 19 kDa protein in the fraction eluted with 250 mM imidazole (Figure 45A). The elution fraction accounts for 93% of esterase activity compared to the activity of the soluble cell extract. The remaining (7%) esterase activity is present in the fraction eluted with 50 mM imidazole, which agrees with the faint TesAH6 protein band observed in the 50 mM fraction on an SDS PAGE gel (Figure 45A). The results of the esterase activity assay and SDS PAGE analysis led us to conclude that recombinant TesAH6 was successfully purified. However, in the purified TesAH6 sample, there appears to be an additional ca. 22 kDa protein, in much lower amounts than TesAH6 (Figure 45B). Matrix-assisted laser dissociation-ionization mass spectrometry (MALDI MS) was used to examine whether these two species are one protein or two different proteins. Prior to protein identification by MALDI MS, samples of both bands were collected from SDS PAGE and hydrolysis of proteins with trypsin was performed. The peptides obtained were identified by means of MALDI MS and the *P. aeruginosa* PA01 proteome was screened in order to attribute them to their respective protein(s). The results show identical peptides detected in both protein bands, which were furthermore characterised as TesA peptides. Therefore, we can conclude that TesAH6 was successfully purified in milligram amounts to high homogeneity.

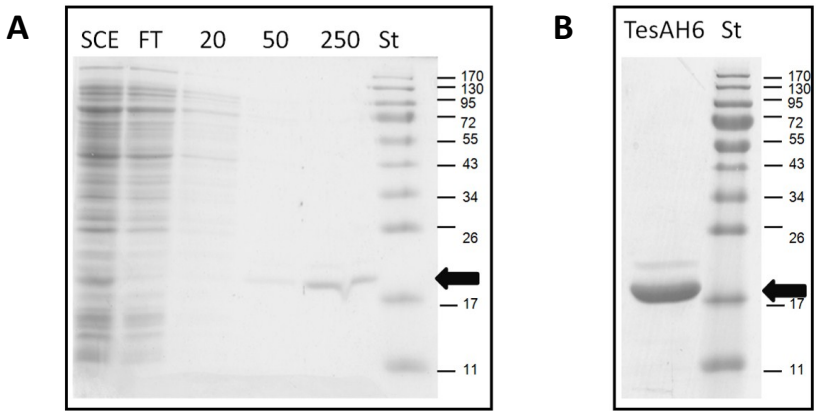


Figure 45: Purification of recombinant TesAH6. **A)** Coomassie-stained SDS PAGE gel (14%) of TesAH6 purification on a Ni-NTA XK16 chromatographic column (Pharmacia). SCE = soluble cell extract of TesAH6 expressed in *E. coli* BL21(DE3) from the pET22-TesAH6 plasmid. FT = flow trough, 20, 50 and 250 = fractions eluted with Ni-NTA purification buffers containing 20 mM, 50 mM and 250 mM imidazole, respectively (Chapter 2.5), Molecular weights of protein standard (St) in kDa are indicated on the right. **B)** Coomassie-stained SDS PAGE gel (14%) of the concentrated TesAH6 purification fraction eluted with 250 mM imidazole (Figure 36A).

After desalting and concentrating the 250 mM elution fraction by ultrafiltration, 7.5 mg of highly homogeneous TesA protein (Figure 45B) from 1 l of bacterial culture was obtained. Pure TesAH6 maintains full esterase activity in Tris-HCl buffer (100 mM, pH 8) after storage at 4˚C for one week. For longer periods, TesAH6 was stored at -20˚C in the presence of 20% (v/v) glycerol as a cryoprotectant. The purified TesAH6 was used for further biochemical and physical and structural characterisation.

4.4.2.2. Temperature stability and optimum of TesA

The optimum catalytic temperature and thermal stability of enzymes are important parameters for biochemical characterization and to study their structure-function relationships. Therefore, we were interested to determine at which temperatures TesA is stable and at which temperature it displays the highest enzymatic activity. In both experiments we have used pure TesAH6 and esterase activity was measured with pNPC as a substrate (Chapter 3.11.1). The highest measured activity was defined as 100% activity and other activities were normalised to this value.

The temperature optimum of TesA was determined by measuring the esterase activity after incubation of TesA with the substrate for 5-15 minutes at different temperatures (Figure 46A). The reactions at lower temperatures were performed longer than the reaction at higher temperatures due to decrease of hydrolysis rate with decrease of temperature. But all activities were expressed per 1 min before relative activities were calculated. TesA displays maximum activity at 35˚C, decreasing already at 30˚C to 40% of the activity. Its activity at 40˚C decreases to 25%, and with higher temperatures, it rapidly decreases further. Activities at lower temperatures (5-25˚C) are very low, less than 20%. These results show a narrow optimal temperature for TesA at 35˚C.

The thermal stability of TesA was determined by measuring esterase activity (pNPC) after incubating the enzyme for 30 minutes at various temperatures. Although the optimal temperature of TesA is 35˚C rather than 30˚C, esterase activities in these experiments were measured at 30˚C due to the

relatively high level of spontaneous pNPC substrate hydrolysis at higher temperatures. The results demonstrate the stability of TesA in a range from 5˚C to 40˚C, where it retains more than 80% of its activity (Figure 46B. Moreover, TesA retains less than 10% activity after incubation at 45˚C. Furthermore, longer incubation (1 h) of TesA at 30˚C results in a decrease of activity to 50% (data not shown). Therefore, we conclude that TesA is a rather thermally unstable enzyme and should be carefully handled to maintain its full activity.

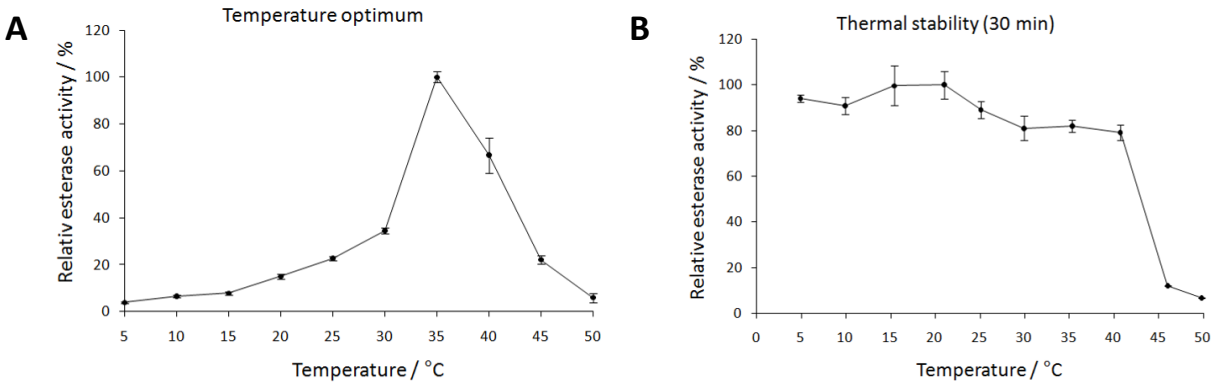


Figure 46: Temperature optimum and thermal stability for TesAH6 measured with an esterase activity assay. Esterase activity assays were performed by incubating 5 µl of TesA (0.013 mg/ml) with 1 ml or 300 µl of substrate for determination of temperature optimum or stability, respectively. For determination of thermal stability, the continuous increase of A_{410nm} was recorded. For determination of optimal temperature, the A_{410nm} was measured after incubation of TesA with the substrate for 5-15 minutes at various temperatures. The activities were normalised to the highest measured activities, namely the activities at 35˚C and 20˚C for temperature optimum and thermal stability, respectively. The results are means of two measurements performed in triplicate.

4.4.2.3. TesA is an esterase rather than a lipase

For a more precise definition of substrate specificity for TesA, an enzyme activity screen with p-nitrophenyl esters of saturated unbranched fatty acids with different chain lengths was performed (Figure 47). The results show the highest activity of TesA toward p-NP pentanoate ($pNPC_5$). Furthermore, TesA hydrolyses pNP esters of butyric, decanoic and dodecanoic acids with high activity. A significant decrease of TesA activity toward pNP esters of fatty acid containing 14 or more carbon atoms was observed. It is known that esters with short-chain fatty acids are preferred by esterases, whereas esters of long-chain fatty acids are preferred by lipases [296]. This preference for short-chain esters and inefficiency at hydrolysing long-chain fatty acid esters classify TesA in the true esterase family [47].

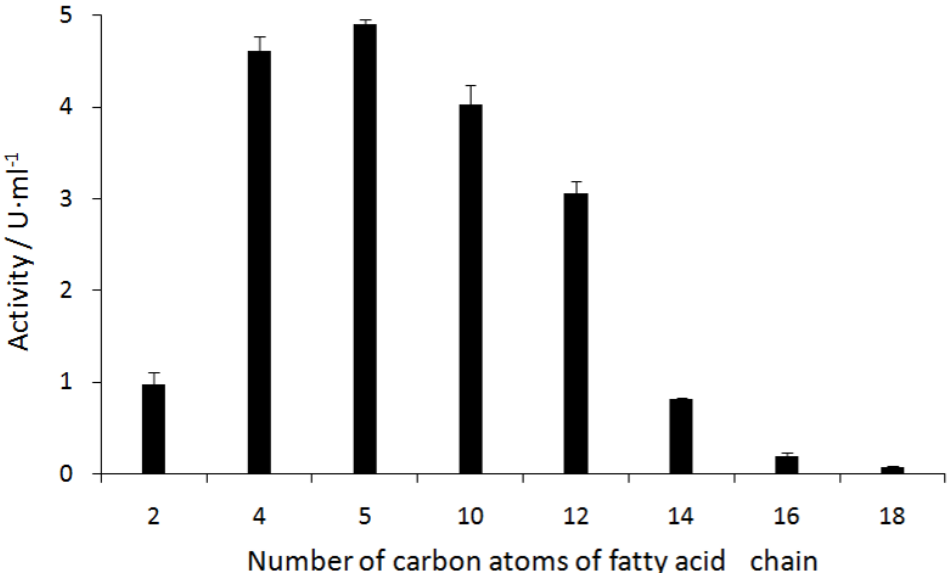


Figure 47: TesA activity measured with various *p*-nitrophenyl fatty acid esters. The assay was performed with 2.5 µl pure TesA (50 µg/ml) per 200 µl substrate solution (5% v/v of 8 mM substrate dissolved in DMSO combined with 100 mM KP_i buffer, pH = 7.5 supplemented with 5 mM sodium deoxycholate and 0.01% w/v gummi arabicum) at 30°C, and the increase in A_{410nm} was continuously recorded over 30 min. The results are means of two measurements, each performed in triplicate.

4.4.2.4. TesA is a lysophospholipase A1 but not a thioesterase

A homology search with the TesA sequence (Chapter 4.2.2) indicates multiple putative functions (arylesterase, protease, lysophospholipase and thioesterase) for TesA. Therefore we have experimentally tested this prediction. Measurements of TesA enzymatic activities against a natural LysoPLA substrate and analogues of natural phospholipase A1 (PLA1), phospholipase A2 (PLA2) and thioesterase substrates reveal a dominant LysoPLA1 activity (2.67 *U*/mg; Figure 48). Furthermore, TesA displays respectively five- and 25-fold less PLA1 (0.49 *U*/mg) and PLA2 (0.08 *U*/mg) activity than LysoPLA1 activity. Thioesterase activity was measured with two different acyl-CoA substrates: short-chain thioester (acetyl-CoA) and long-chain thioester (stearyl-CoA). The activity against acetyl-CoA was not measurable and the activity against palmitoyl-CoA was insignificant (<0.01 *U*/mg). The high sequence homology of TesA to *E. coli* TAP, a multifunctional enzyme that exhibits esterase, thioseterase, lysophospholipase and protease activities [3], has been previously described. We were able to demonstrate esterase and modest LysoPLA1 activities for TesA but not thioesterase activity. To examine any additional multifunctionality of TesA, its protease activity against the general protease substrate casein was assayed. The results show that TesA is not able to hydrolyse casein (data not shown); therefore, it cannot be considered a protease.

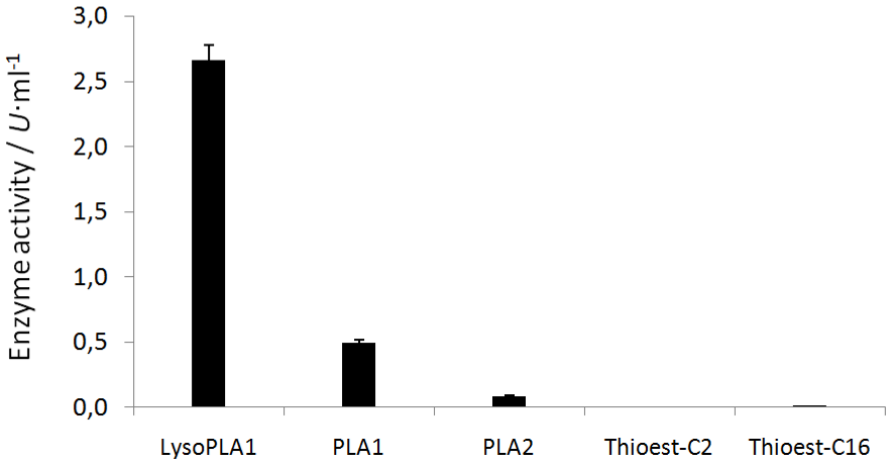


Figure 48: Screen for hydrolytic activities of TesA. LysoPLA1, PLA1 and PLA2 activities were measured with 1-monopalmitoyl phosphatidylcholine, 1-thiopalmitoyl 2-palmitoyl phosphatidylcholine and 1,2-dithiodiheptanoyl phosphatidylcholine as substrates, respectively (Chapter 3.11.4). Thioesterase activities were measured (Chapter 3.11.5) with acetyl-CoA (Thioest-C2) and palmitoyl-CoA (Thioest-C16) as substrates. All reactions, except with the LysoPLA1 substrate, were performed by incubating 2.5 µl pure TesA (128 µg/ml) with 200 µl of the respective substrate solution at 35°C, with the increase in A_{410nm} recorded continuously. The LysoPLA1 activity was measured by incubating 25 µl substrate with 25 µl enzyme solution (128 µg/ml) as described in methods (Chapter 3.11.3). The results are means of two measurements each performed in triplicate.

The results presented here point out evident differences in enzymatic functions for TesA and TAP [3] despite their 59% sequence similarity. Whereas TesA was predicted to be a thioesterase, we have clearly demonstrated that it not a thioesterase. It shows robust esterase and LysoPLA1 activities as well as a minor PLA2 activity. Additionally, the protease activity of TesA was not measurable. The results indicate TesA is an enzyme with a narrower substrate spectrum (esterase and LysoPLA) than the broad substrate spectrum typical for multifunctional enzymes like *E. coli* TAP, which exhibits esterase, thioesterase, lysophospholipase and protease activities [5].

4.4.2.5. Inhibition of TesA

Inhibition studies provide useful data on enzyme structure-function relationships. Additionally, covalent enzyme-inhibitor complexes may provide information about catalytic residues and binding sites in the active sites of enzymes. TesA is predicted to be an active site serine hydrolase with Ser_{30} predicted to serve as a nucleophilic amino acid in catalysis. Therefore, the sensitivity of TesA to inhibitors that target the catalytic Ser, namely PMSF and THL, was tested. Both inhibitors covalently bind to the nucleophilic serine of serine hydrolases, specifically THL (by means of its reactive β-lactone group) and PMSF, with a reactive sulphonyl fluoride group, resulting in irreversible inhibition of enzymatic activity. However, these two inhibitors are specific for different groups of enzymes. THL is a typical pancreatic lipase inhibitor [297] displaying inhibitory activity against microbial lipases as well [298]. PMSF is a general serine protease inhibitor with demonstrated inhibitory activity against a range of bacterial enzymes such as proteases [299], esterases [300] and glycosidases [301].

The residual esterase activity of TesA was measured after incubation of pure TesA for 1 h on ice (Chapter 3.12) with each inhibitor at concentration of 1 mM, which represents an inhibitor molar excess of 3 X 10^5. The results indicate a stronger inhibition of TesA with THL (80.3 ± 0.5%) than PMSF

(65.3 ± 6.8%) under the selected conditions (Figure 49). It is notable that complete inhibition was not reached even though the inhibitors were in high molar excess. Therefore, we have tested the esterase activity of TesA after its incubation overnight with each inhibitor as before. Neither inhibited sample revealed esterase activity, whereas untreated TesA retained its activity. Thus, we conclude that these two irreversible inhibitors interact differently with TesA and they completely inhibit TesA activity after longer incubation of enzyme with the inhibitor. These data support THL and PMSF as potential substances for further crystallographic studies of the substrate binding pocket of TesA.

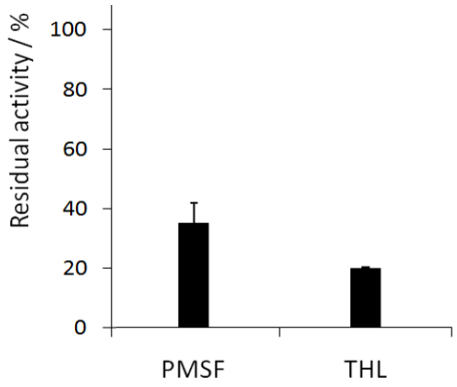


Figure 49: Inhibitory effect of PMSF and THL on TesA. Esterase activity was measured with *p*-NP pentanoate (see esterase assay) after preincubation (1 h, 0°C) of TesA (0.076 µg/ml) with 1 mM PMSF or THL (Chapter 3.12). The residual activity was normalised to the activity of untreated TesA. The results are means of three measurements each performed in triplicate.

4.4.2.6. Annotation of TesA active site

In chapter 4.2.2.2, the active site of TesA was predicted according to its sequence homology with enzymes whose three dimensional structures are available. Amino acids Ser_{30}, Asp_{177} and His_{180} have emerged as the putative catalytic triad residues of TesA. Therefore, the roles of these three residues were proven by exchanging each of them with Ala. Three point mutants ($TesA_{S30A}$, $TesA_{D177A}$ and $TesA_{H180A}$) of TesA were generated by site-directed mutagenesis (Chapter 3.6.1).

The catalytic mechanism of Ser hydrolases is well understood [48, 5]. A hydrogen bond network is composed of the side chain hydroxyl group of Ser and the imidazole ring of His, and furthermore the imidazole ring of His and side chain of Asp are crucial for proper enzymatic function. Therefore, replacing Ser, Asp or His with Ala, whose side chain does not satisfy the physicochemical requirements for assuming a catalytic role, should yield inactive TesA mutants if the prediction of the catalytic triad is reliable. The expression plasmids pET22b-$TesA_{S30A}$, pET22b-$TesA_{D177A}$, and pET22b-$TesA_{H180A}$, which we constructed with the desired mutations and confirmed by nucleotide sequencing, were used for overexpression of TesA mutants. Catalytically active TesA was expressed from pET22b-TesA vector, and pET22b was used as a negative control. The expression was carried out in *E. coli* BL21(DE3) under autoinduction conditions for 20h. SDS PAGE analysis of overexpression reveals an additional overexpressed band of ca. 19 kDa following expression of TesA, $TesA_{S30A}$, $TesA_{D177A}$ and $TesA_{H180A}$ in comparison to the empty vector (pET22b) control (Figure 50). Furthermore, from SDS PAGE (Figure 50), we noted that the expression level of TesA mutants is higher than that of native TesA. To prove this observation, however, it is necessary to purify all proteins expressed under identical conditions and quantify the expression levels according the amounts of pure enzyme.

Furthermore, the soluble cell extracts of TesA, $TesA_{S30A}$, $TesA_{D177A}$ and $TesA_{H180A}$ were compared mutually and with the empty vector control by means of an esterase activity assay (pNPC). The

results (Figure 50) clearly indicate lack of enzymatic activity for all three TesA mutants in comparison to the native TesA. The level of esterase activity for TesA mutants is nearly the same as the empty vector control, which strengthens the conclusion that the mutations (S30A, D177A and H180A) generated in TesA resulted in enzyme inactivation. Taken together, the reliable prediction of Ser30, Asp177 and His180 as the TesA catalytic triad and the successful expression of those mutants that were inactive in the esterase activity assay allow us to draw the conclusion that these three amino acids are indeed crucial for the enzymatic activity of TesA. It should be noted that the introduced amino acid changes could disturb the tertiary structure of TesA, which may led to the expression of incorrectly folded and therefore enzymatically inactive TesA. However, incorrectly folded proteins are susceptible to protease degradation or tend to accumulate as insoluble aggregates (inclusion bodies). TesA mutants were not present in inclusion bodies (data not shown) and their expression was even higher than that of catalytically active TesA. Therefore, it is not likely that the induced amino acid changes drastically disturbed the TesA structure to result in its inactivation. This set of experiments encourages us to conclude that Ser30, Asp177 and His180 are members of the catalytic triad of TesA.

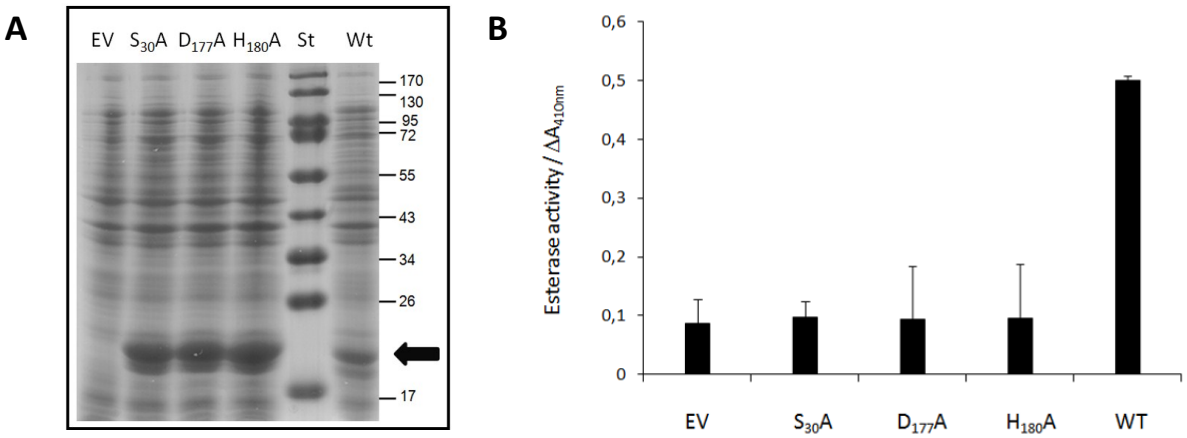


Figure 50: Mutation of putative active site residues of TesA. **A)** Coomassie-stained SDS PAGE gel (14%) of TesA (Wt), $TesA_{S30A}$ ($S_{30}A$), $TesA_{D177A}$ ($D_{177}A$) and $TesA_{H180A}$ ($H_{180}A$) expressed in *E. coli* BL21(DE3) under autoinduction conditions (Chapter 3.1.1). Molecular weights of protein standard (St) in kDa are indicated on the right. EV = empty vector control pET22b. 0.15 U_{Cell} of total cell lysate was loaded on the gel. The black arrow indicates overexpressed TesA. **B)** Esterase activity assay (pNPC) was performed with 10 µl soluble cell extract (0.1 U_{Cell}) per 200 µl substrate solution, and the increase in A_{414nm} was monitored continously over 15 min. The results are means of three measurements, each performed in triplicate.

4.4.2.7. Expression and cellular localization of TesA in *P. aeruginosa* PA01

To gain the knowledge about the physiological function of TesA, it was expressed in a homologous host (*P. aeruginosa* PA01) and the esterase activity of each cell compartment was examined. In Chapter 4.2.3, we have predicted a signal peptide in TesA that indicates its possible transport across the inner membrane. To experimentally prove this hypothesis, it is important to separate extracellular, periplasmic, cytoplasmic and membrane proteins from each other. Therefore, TesA was expressed from the pBBR-TesA plasmid, where it is under the control of the constitutive promoter P_{lac}. The growth conditions were set up to allow relatively low expression of TesA to avoid oversaturation of the protein transporting systems but allow a sufficient amount of TesA for its

useful detection by esterase activity assays. Therefore constitutive expression was carried out by growing *P. aeruginosa* PA01 transformed with pBBR-TesA in LB medium for 16 h (standard conditions). At this time point, cells were in stationary phase where proper function of the protein transport mechanism could be expected. The cell compartments (extracellular space, periplasm, membranes and cytoplasm) of strains expressing TesA and empty vector (pBBR1mcs-3) were separated (Chapter 3.14.2) and the esterase activity was measured by two methods. A) The total esterase activity of the intact cell fractions was measured using a liquid esterase assay (pNPC). B) Furthermore, the cell fractions were subjected to a zymographic analysis using 4-methylumbelliferyl butyrate (MUB) as a substrate (chapter 3.11.8). In previous experiments, purified TesA was successfully reactivated after separation on an SDS PAGE gel by incubating the gel in renaturation buffer (data not shown). The hydrolytic activity of TesA was demonstrated by overlaying the gel with the pro-fluorescent substrate MUB that is commonly used to examine esterase activity [172]. In localisation experiments, the same conditions for zymographic detection of TesA activity were applied, including pure TesA as a control.

The liquid esterase assay (pNPC) (Figure 51A) indicates a 2.5-fold increase in activity in the periplasmic fraction of the TesA-expressing strain in comparison to the empty vector strain. Furthermore, a 25% increase in esterase activity was observed in the cytoplasmic fraction when comparing the TesA-expressing strain to the empty vector control. The esterase activities for membrane fractions of *P. aeruginosa* expressing TesA and empty vector do not differ significantly (data not shown in figure 51A due to simplicity). They were much higher than those of periplasmic fraction, EV = 617% and TesA = 627%, normalised to the activity of the periplasmic fraction of the *P. aeruginosa* expressing TesA. In the outer membrane of *P. aeruginosa* PA01 esterase EstA is located, thus the activity measured in membrane fractions are likely a result of EstA.

As one may assume that *P. aeruginosa* PA01 constitutively expresses other esterases beside TesA (some have been described in the literature and some were discovered in the course of this PhD work), we tried to detect TesA specifically in each cell compartment. Due to a lack of specific antibodies against TesA, we have applied a zymographic method for detection of TesA. The zymographic analysis of *P. aeruginosa* PA01 cell compartments expressing TesA or carrying an empty vector showed an activity of a ca. 20 kDa periplasmic protein in both strains (Figure 51B). The activity measured in the periplasmic fraction of the TesA-expressing strain is significantly stronger comparing to the respective empty vector control. Furthermore, we noticed a low activity present in the cytoplasmic fraction of the TesA-expressing strain that is the same size as one in the periplasmic fraction. The size of the protein active in the zymogram (Figure 51B) corresponds to the size of TesA; therefore, it may be ascribed to TesA. Furthermore, the data obtained by the liquid esterase assay are in agreement with the zymogram activity profile of TesA. Taken together, the significant increase in esterase activity of the periplasmic fraction after expression of TesA and the strong activity of TesA in the periplasmic fraction of the TesA-expressing strain observed in the zymogram, we can conclude a periplasmic, but not extracellular localization for TesA. In liquid assay, we measured a slightly increased esterase activity of the cytoplasmic fraction from the strain expressing TesA compared to the corresponding empty vector control. The faint TesA band was observed in the cytoplasmic fraction of the TesA-expressing strain obtained in the zymogram. Both phenomena may be explained by incomplete extraction of periplasmic proteins from cells (likely) or by accumulation of TesA in the cytoplasm due to insufficient protein transport across the inner membrane (not likely).

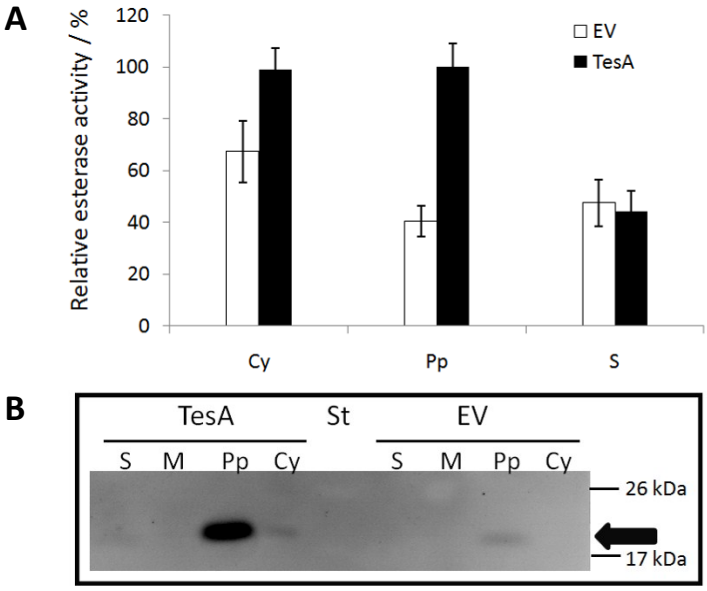


Figure 51: Subcellular localisation of TesA in *P. aeruginosa* PA01. **A)** A liquid esterase activity assay (pNPC) was performed incubating 5 µl of each cellular fraction (equivalent to 0.025 U_{Cell}) with 180 µl of substrate solution, and the increase in A_{410nm} was recorded continuously during a period of 10 min. The results are means of two measurements, each performed in triplicate. **B)** Section of the zymogram gel presenting the esterase activity of cell compartments from *P. aeruginosa* PA01 transformed with pBBR-TesA (TesA) and pBBR1mcs-3 (EV, empty vector). Molecular weights of protein standard (St) in kDa are indicated on the right. After SDS PAGE electrophoresis (14% gel), the gel was subjected to renaturation conditions, and esterase activity was monitored under UV light using the fluorescent substrate 4-methylumbelliferyl butyrate.

TesA purified to homogeneity was subjected to crystallisation screens that resulted in several conditions under which protein monocrystals were obtained. We have observed a preference for TesA to crystallise in the presence of polyethylene glycol in low ionic strength acidic sodium citrate buffer. The protein content and quality of crystals were proven in an X-ray beam, and currently, the collection of diffraction signals and solving of the three-dimensional structure of TesA are in progress.

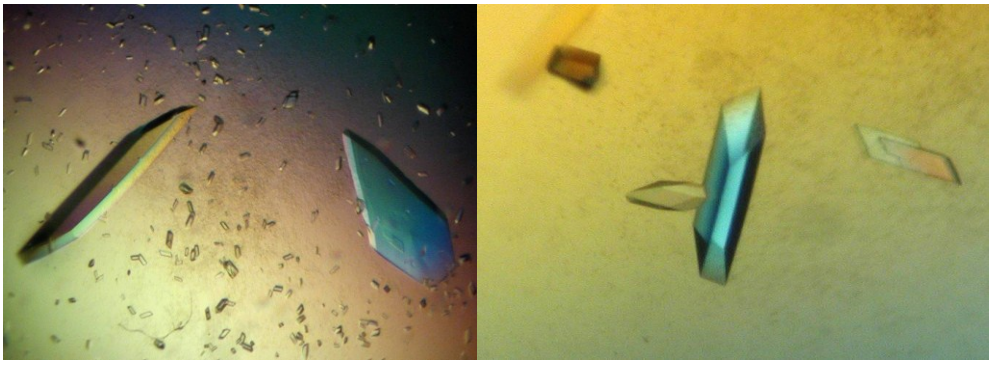


Figure 52: Crystals of TesA. Protein crystals were obtained by mixing the same volume of TesAH6 solution (10 mg/ml in 50 mM Tris-HCl buffer, pH = 8) with sodium citrate buffer (50 mM, pH 4.5) supplemented with 20% (w/v) polyethylene glycol (Mw = 3350, PEG3350). Crystallisation was performed in 1.6 µl hanging drops at 19˚C.

Summary

4.4.2 TesA, a periplasmic GDSL-hydrolase from P. aeruginosa with esterase and lysophospholipase A activities

TesA was named thioesterase A according to its pronounced sequence homology to the thioesterase from *E. coli* (TAP) [3]. Both TesA and TAP contain a conserved GDSL peptide around the catalytic serine that is characteristic of the GDSL-hydrolase family [4]. Indeed, multiple sequence alignments of TesA with known GDSL-hydrolases reveal conservation of all four active site motifs (block I, II, III and V) characteristic of GDSL-hydrolases (Figure 17). Therefore, the catalytic triad (Ser30, Asp177 and His180) and oxyanion hole (Gly67 and Asn96) residues of TesA were predicted. To experimentally prove whether Ser30, Asp177 and His180 are the catalytic triad residues of TesA, those amino acids were exchanged against alanine, which should result in the inactivation of TesA. The overexpression of TesA point mutants (Figure 50A) that were unable to hydrolyse an esterase substrate (Figure 50B) confirmed the prediction that Ser30, Asp177 and His180 represent the catalytic triad of TesA.

Heterologous overexpression of TesA in *E. coli* has resulted in the production of a ca. 20 kDa TesA protein that displays esterase activity. Furthermore, recombinant TesA tagged with a His_6-tag on its carboxy-terminal end was purified on a Ni-NTA column to high homogeneity. The pure enzyme exhibits strong esterase and LysoPLA1 activity, modest PLA1 activity, low PLA2 activity and no thioesterase and protease activities. It seems that although sequence homology between TesA and *E. coli* TAP is high (62% similarity, 44% identity), their substrate specificity is dissimilar. Whereas TAP displays esterase, thioesterase, lysophospholipase A1 and protease activities [5], TesA has only esterase and lysophospholipase A1 activities. Furthermore, a screen for TesA activity with long- and short-chain fatty acids esters revealed significant hydrolysis of esters with dodecanoic (and shorter fatty acids) as opposed to longer fatty acid esters. Such substrate specificity is typical for esterases rather than for lipases.

Using purified TesA, its temperature optimum was determined to be 35˚C (Figure 46A). Furthermore, TesA is stable for 30 min at temperatures up to 40˚C but retains less than 10% activity after incubation for 30 min at 45˚C (Figure 46B). Therefore, we conclude that TesA is a thermolabile rather than thermostable enzyme.

Treatment of TesA with the irreversible catalytic Ser targeting inhibitors PMSF (serine protease inhibitor) and THL (pancreatic lipase inhibitor) reveals 65.3% and 80.3% enzyme inhibition, respectively. These data confirm that TesA belongs to the large group of serine hydrolases.

Analysis of the TesA sequence identified a putative signal peptide; therefore, it is expected to be translocated across the inner membrane to the periplasm or extracellular space. This hypothesis was examined by detection of TesA in discrete cell compartments of *P. aeruginosa* PA01. Therefore, TesA was expressed in *P. aeruginosa* PA01 and extracellular, periplasmic, cytoplasmic and membrane proteins were separated. Furthermore, the total esterase activity of each cell fraction was measured, and specific detection of TesA in each cell fraction was performed by

means of a zymographic method. The total esterase activity of the periplasmic, but not extracellular, fraction increased upon expression of TesA (compared to empty vector control) (Figure 51A). The zymographic analysis indeed indicates the presence of TesA in the periplasmic and not the extracellular fraction. Furthermore, TesA activity also was observed in EV expressing strain, which indicates constitutive expression of TesA in *P. aeruginosa* PA01. These data revealed that TesA, a novel esterase/LysoPLA1 of *P. aeruginosa* PA01, is a periplasmic, not an extracellular, protein.

4.4.3. Membrane bound phospholipase B, PlbF of *P. aeruginosa* PA01

The third enzyme examined in detail in the course of this PhD work is the putative phospholipase A, PlbF. Interestingly, it is homologous to three groups of physiologically relevant hydrolases: lipases from psychotrophic organisms, bacterial enzymes involved in catabolism of aromatic compounds and mammalian hydrolases participating in lipid signal pathways (Table 13). Therefore, we have analysed PlbF, a putative phospholipase A from *P. aeruginosa* PA01 with predicted signal peptide. To experimentally demonstrate hydrolytic activity of PlbF, cells expressing PlbF and purified PlbF were examined by means of esterase, lipase and PLA activity assays. Furthermore, its subcellular localization was explored in detail. A *plbF* negative mutant strain was constructed with the aim of examining physiological function of PlbF in *P. aeruginosa* PA01.

4.4.3.1. Substrate specificity of PlbF, a putative lipase of *P. aeruginosa* PA01

The initial biochemical characterisation of PlbF was carried out with total cell lysate and not with purified PlbF, due to difficulties in purification of PlbF that will be discussed later. Therefore, PlbF was expressed in *E. coli* BL21(DE3) as described previously (Chapter 4.3.2) and its esterase activity was confirmed (Figure 40). Enzymatically active (esterase activity assay) total cell extract of PlbF expression was furthermore compared to the empty vector control (*E. coli* BL21(DE3) transformed with pET22b) using PLA1, PLA2 and thioesterase assays. Thioesterase activities were measured with two different substrates, acetyl- and palmitoyl-CoA, which differ in the length of fatty acids bound to Coenzyme A. The results show that cells expressing PlbF are able to hydrolyse PLA1, PLA2 and palmitoyl-CoA with much higher rates compared to empty vector controls (Figure 53). Furthermore, the thioesterase activity of PlbF measured with acetyl-CoA as a substrate is very low. Although the thioesterase-C16, PLA1 and PLA2 activates of PlbF are mutually similar, they are approximately one order of magnitude lower than the corresponding esterase activity (data not shown). Therefore, we can conclude that PlbF is a novel hydrolase with pronounced esterase activity and auxiliary thioesterase, PLA1 and PAL2 activities.

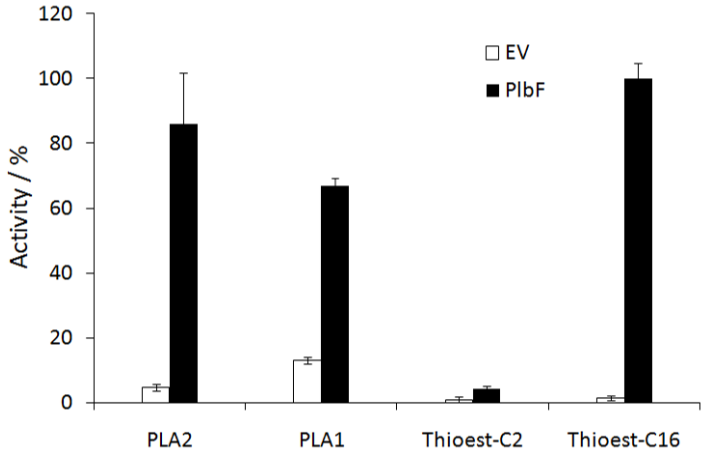


Figure 53: Lipolytic activities of PlbF. PLA1 and PLA2 activities were measured with 1-thiopalmitoyl 2-palmitoyl phosphatidylcholine and 1,2-dithio-diheptanoyl phosphatidylcholine as substrates, respectively, and thioesterase activities were measured with acetyl-CoA (Thioest-C2) and palmitoyl-CoA (Thioest-C18) as substrates. Reactions were performed by incubating 10 µl (0.15 U_{Cell}) total cell lysate of *E. coli* BL21(DE3) cells expressing PlbF (autoinduction conditions) and respective empty vector controls with 200 µl of the above listed substrate solutions at 30°C. The increase in A_{414nm} was continuously recorded over 10 minutes. The results are means of two measurements, each performed in triplicate.

4.4.3.2. Inhibition study of PlbF

To explore if PlbF is a Ser hydrolase, two inhibitors (PMSF and THL) that specifically bind to the nucleophilic serine in the active sites of serine hydrolases were tested. Furthermore, EDTA as a bivalent cation chelator was used to test whether PlbF belongs to the group of metalloenzymes. PMSF and THL were previously used for inhibition of TesA (Chapter 4.4.2), where they were described in detail. Some bacterial esterases required metal ions for enzymatic activity [47]; therefore, the metal chelator EDTA might inhibit their activity. Metal ions have a structural [302] and functional role [303, 304] in enzymes, thus removing ions by EDTA induces global changes in protein folding (protein denaturation) or produces an incomplete active site incapable of catalysis.

In the inhibition experiments, total cell lysate of *E. coli* BL21(DE3) expressing PlbF (Chapter 4.3.2) from the pET22b-PlbF plasmid was used. Previously, overexpression of PlbF was confirmed by means of esterase activity comparing the empty vector strain with the PlbF expression strain. In this study, the PlbF samples were incubated for 3 h on ice with a corresponding concentration of EDTA (10 mM), THL (5mM) and PMSF (1 mM), followed by measurements of esterase activity (Figure 54). The PlbF samples in the absence of inhibitor, treated the same as the inhibited samples, were used to calculate the residual activity after inhibition. The results display ca. 20% inhibition of PlbF by EDTA and ca. 60% inhibition by THL. Moreover, the esterase activity of PlbF is completely inhibited after treatment with PMSF. Because the activities of PlbF were not affected by DMSO (the solvent for THL) or propan-2-ol (the solvent for PMSF), which were present in the inhibition samples, we can conclude that the decrease in PlbF activity is due to the inhibitory effects of THL and PMSF. These results confirm the role of a nucleophilic Ser in PlbF catalysis, which is in keeping with the prediction of PlbF as a serine hydrolase. Furthermore, the observed differences in inhibition of PlbF with PMSF (strong inhibition) and THL (partial inhibition) suggest that PlbF is an esterase rather than a lipase. The resistance of PlbF to EDTA suggests that PlbF does not required metal ions for its proper catalysis.

These rough data, obtained using a cell lysate instead of a pure enzyme, clearly show that PMSF may be used for complete inhibition of PlbF activity.

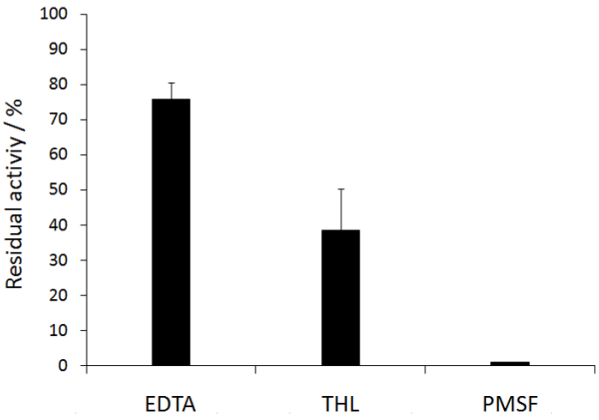


Figure 54: Inhibition of PlbF with EDTA, THL and PMSF. Esterase activity was measured with pNPC after preincubation of PlbF (1.5 U_{Cell}/ml of total cell lysate) for 3 h on ice with 1 mM PMSF, 5 mM of THL and 10 mM of EDTA. The activity was measured with 6 µl (0.09 U_{Cell}) PlbF sample per 150 µl substrate solution, and the increase in A_{410nm} was continuously recorded over nine minutes. Activity was normalised to the activity of untreated PlbF samples. The results are means of three measurements.

4.4.3.3. Solubilisation and purification of PlbF

It has been mentioned previously that purification of PlbF was difficult rather than straightforward. Although, overexpression of PlbF and PlbF tagged with the hexa-His peptide on its carboxy-terminal end (PlbFH6) in *E. coli* and *P. aeruginosa* PA01 was useful for detection of PlbF by an esterase activity assay, the amount of protein was not sufficient to allow for efficient visualization by standard SDS PAGE analysis. It seems that homologous expression yields higher amounts of PlbFH6 than expression in *E. coli* DH5α (controlled by P_{lac}) or in *E. coli* BL21(DE3) (controlled by P_{T7}) (data not shown). Furthermore, initial experiments in *E. coli* and *P. aeruginosa* indicated lack of PlbF and PlbFH6 in inclusion bodies and soluble fractions, cytoplasmic and periplasmic cell compartments (data not shown). The reason is probably a membrane association of PlbFH6. Therefore, attempts to purify PlbFH6 from total cell lysates of *E. coli* BL21(DE3) and *P. aeruginosa* PA01 cultures failed (data not shown).

As in the membrane-associated PlbFH6, the His6-tag could be buried in the membrane, hindering its interaction with the Ni^{2+} ions of the Ni-NTA material. To test this hypothesis we tried to a) localise PlbF and b) purify PlbFH6 from the membrane. Two detergents were selected in attempt to release PlbF from membranes, namely Triton X-100 (TX-100) and sodium N-lauroyl sarcosine (NLS). Both detergents are described as reagents generally used for solubilisation of proteins from membranes after overexpression in *E. coli* [305]. However, they have different physicochemical properties. NLA is an ionic detergent, whereas Triton is a non-ionic detergent. The minimal detergent concentration at which it forms micelles, called the critical micellar concentration (CMC) differs for TX-100 and NLS. The CMC of TX-100 is ca. 0.03% (w/v) (0.5 mM), whereas the CMC of NLS is around 0.5% (w/v) (15 mM), with the caveat that it is highly dependent on the ionic strength and composition of the solution. These differences in detergent properties imply differences in membrane-detergent and protein-detergent interactions. Therefore, differences in protein solubilisation and activation/inactivation effects could be expected for these two detergents. In solubilisation and purification experiments, the *P. aeruginosa* PA01 strain was favoured over the *E. coli* strains due to high yield of PlbFH6 expression, simplicity of expression (no need to induce expression) and the fact

that a homologous host may be more suitable for expression of membrane proteins than a heterologous host.

Membranes from *P. aeruginosa* PA01 transformed with pBBR-PlbFH6 and grown overnight in LB medium (Chapter 3.1.2), were isolated (Chapter 3.14.2). Furthermore, equal amounts of membrane (5.5 U_{Cell}) were incubated with 200 µl of 0.5%, 1% and 2% (w/v) concentrations of NLS and Triton (detergents were dissolved in Tris-HCl buffer, 100 mM, pH 8). The solubilisation was performed by gently vortexing samples for 30 min at room temperature. After incubation, esterase activities of the samples were measured in order to determine whether the activity of PlbFH6 was affected by detergents (Figure 55A). The results indicate a minor effect of TX-100 on the esterase activity of PlbFH6 at all three concentrations (which are above CMC). It is notable that Triton at lower concentrations inactivates PlbFH6 more strongly than at higher concentrations. In contrast to Triton, NLS has a stronger effect on PlbFH6 activity. At 0.5% NLS (near its CMC), strong inactivation (74%) of PlbF was observed. At concentrations above the CMC of NLS (1% and 2%), an activating effect on PlbF (ca. 60% activation) was observed. After analysing the influence of NLS and TX-100 on the esterase activity of PlbF, the influence on solubilisation of PlbFH6 was examined. Membrane samples after treatment with detergents (30 min, room temperature, gentle vortexing) were centrifuged for 3 h at 20000x*g*. The pellets (membranes) and supernatants (solubilised proteins) were then analysed by means of SDS PAGE and Western blot (using anti-His6-tag antibodies) for solubility of the PlbFH6 protein. We present only the results obtained with TX-100 because it has no significant influence on PlbF activity, which highlights this detergent as a good reagent for purification of PlbFH6. On the Western blot, a PlbFH6 signal was detected in the soluble fraction of membranes treated with 0.5%, 1% and 2% of TX-100 but not in the insoluble fractions (Figure 55B). In the case of untreated samples it is opposite, on the Western blot, a signal for PlbFH6 is present in the insoluble but not the soluble fraction of negative control samples (membranes treated with buffer lacking TX-100, 0% Triton).

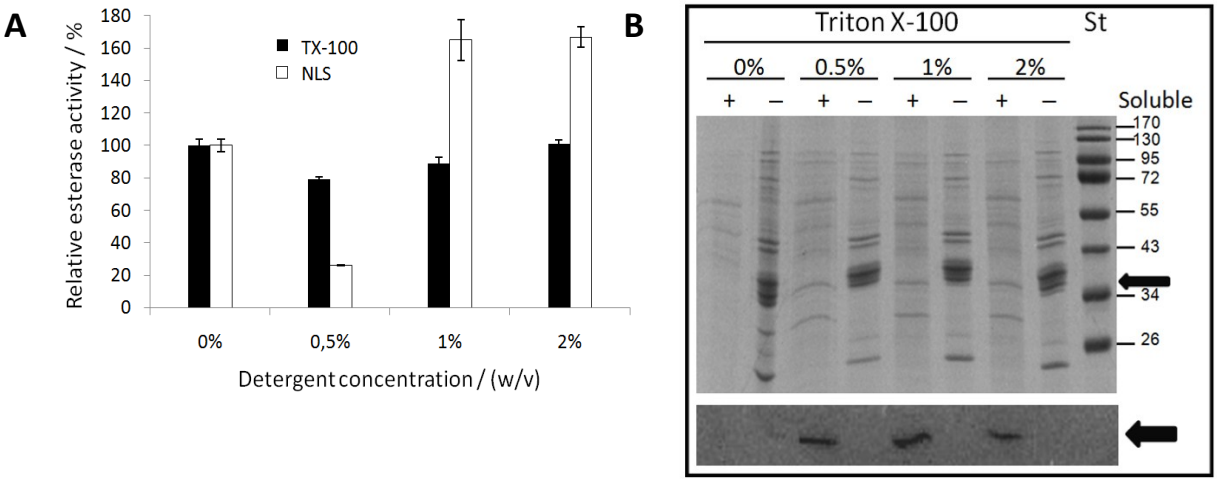


Figure 55: Solubilisation of PlbFH6 from *P. aeruginosa* membranes. **A)** The esterase activity of the membrane fraction of *P. aeruginosa* PA01 expressing PlbFH6 (20 U_{Cell}/ml), treated for 30 minutes at room temperature with buffer (Tris-HCl, 100 mM, pH = 8) supplemented with 0%, 0.5%, 1% and 2% (v/v) Triton X-100. The esterase activity assay was performed with 5 µl sample per 200 µl substrate solution, and the increase in A_{410nm} was continuously recorded over 2 min. The results are means of three measurements. **B)** Coomassie-stained SDS PAGE (14%) (upper panel) and respective Western blot detected with anti-His6-tag antibodies (lower panel) for soluble (+) and insoluble (-) membrane fractions of *P. aeruginosa* PA01 expressing PlbFH6 and treated with TX-100. The equivalent of 0.1

U_{Cell} of each sample was loaded on the gel. Molecular weights of protein standard (St) in kDa are indicated on the right. Black arrows indicate PlbFH6.

Apparently, the Western blot signal for soluble PlbFH6 is significantly stronger than the signal of membrane-bound PlbFH6 (Figure 55B). The observed difference in immunoreactivity for membrane-associated PlbFH6 and soluble PlbFH6 suggests an inaccessibility of the His6-tag in membrane-bound PlbFH6. SDS PAGE analysis revealed the presence of PlbFH6 in the soluble but not insoluble fraction of membranes treated with TX-100 (Figure 55B). Furthermore, PlbFH6 was observed in a Coomassie-stained SDS PAGE gel in the insoluble but not soluble fraction of untreated membranes (negative control; Figure 55B). Taken together, we can conclude that TX-100 successfully solubilises PlbFH6 from membranes at all tested concentrations, and soluble PlbFH6 retains its full activity after solubilisation with 2% (v/v) TX-100.

The advantageous PlbFH6 solubilisation from membranes using TX-100 was used for purification of PlbFH6 on a Ni-NTA column. The expression of PlbFH6 in *P. aeruginosa* PA01 and solubilisation of PlbFH6 from membranes was performed as described above. Solubilised PlbFH6 membranes were subjected to a Ni-NTA column (gravity flow, 3 ml material) and PlbFH6 was eluted with purification buffers contain 20 mM, 60 mM and 250 mM imidazole (Chapter 3.16) supplemented with 2% (v/v) TX-100 to maintain PlbFH6 in a soluble form. The SDS PAGE analysis reveals one predominant ca. 35 kDa protein present in fractions eluted with 60 mM and 250 mM imidazole (Figure 56). Furthermore, measurements of esterase activity reveal more than 95% of activity (compared to the sample loaded on the column) present in the 60 mM and 250 mM elution fractions. The residual esterase activity (less than 5%) was measured in the flow through fraction. Because PlbFH6 was not observed on the SDS PAGE gel in the flow through fraction, this activity may be attributed to other membrane-bound esterases of *P. aeruginosa* PA01 rather than PlbFH6. Therefore, we can conclude that Triton-solubilised PlbFH6 binds to the Ni-NTA resin with satisfactory affinity and this method may be applied for purification of wild type PlbFH6. The purified PlbFH6 displays low stability after freezing at -20˚C or storage for longer times (one week) at 4˚C in the presence or absence of 20% (v/v) glycerol. We have observed that the protein concentration in the solution and presence of detergents strongly influenced PlbF activity. Thus, the specific activity of PlbFH6 measured with esterase substrate (pNPC) was in the range of 50-1000 U/mg. These factors prevented its detailed biochemical characterisation. Therefore, to fully characterise PlbFH6, it is necessary to discover conditions to maintain PlbFH6 stably in solution.

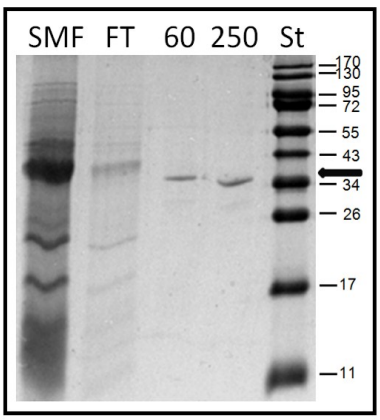


Figure 56: Purification of recombinant PlbFH6. Coomassie-stained SDS PAGE gel (14%) for PlbFH6 purification on a gravity flow (3 ml) Ni-NTA column (Chapter 3.16). SMF = solubilised membrane fraction from PlbFH6 expression, FT = flow through, 60 and 250 = fractions eluted with buffers contain 60 mM and 250 mM imidazole, respectively (Chapter 2.5), Molecular weights of protein standard (St) in kDa are indicated on the right. Black arrow indicates PlbF.

4.4.3.4. Expression and subcellular localization of PlbF in *P. aeruginosa* PA01.

We have predicted a putative signal peptide in PlbF that indicates its periplasmic or extracellular localization (Chapter 4.2.3). Furthermore, transmembrane helices were predicted in PlbF (Chapter 4.2.3) suggesting its membrane localisation. Our previous hints (from purification experiments) about membrane localisation of PlbF were further examined. Therefore, the supernatant and total cell lysate were assayed for the presence of PlbFH6 after its expression in *P. aeruginosa* PA01 from the pBBR-PlbFH6 plasmid in TSB medium (standard growth conditions). The growth of *P. aeruginosa* PA01 transformed with pBBR-PlbFH6 and pBBR1mcs-3 (empty vector control) was compared (Figure 57), and samples at five growth points, ranging from early logarithmic phase to early stationary phase, were analysed. Although protein transport across the dual-membrane envelope of *P. aeruginosa* PA01 is a complex process that employ various mechanisms [306], it is generally accepted that *P. aeruginosa* PA01 secretes a number of proteins at high cell densities [8]. Therefore, cultures at different growth stages were analysed in order to rule out intracellular accumulation of PlbF due to lack or overloading of transport mechanisms. Proteins from the supernatant were concentrated by means of TCA precipitation (Chapter 3.18.2) and total cells were resuspended directly in SDS PAGE sample buffer (Chapter 2.5). These samples were subjected to Western blot analysis with anti-His6-tag antibodies. The results indicate the absence of PlbFH6 in the extracellular space of *P. aeruginosa* PA01, but it was detected as a cell-associated protein (Figure 57). It was observed that PlbFH6 is expressed already during the early logarithmic growth phase. Moreover, Western blot signals of low molecular weight proteins (below the signal of PlbFH6) whose intensities gradually increase following PlbFH6 expression indicate degradation of PlbFH6.

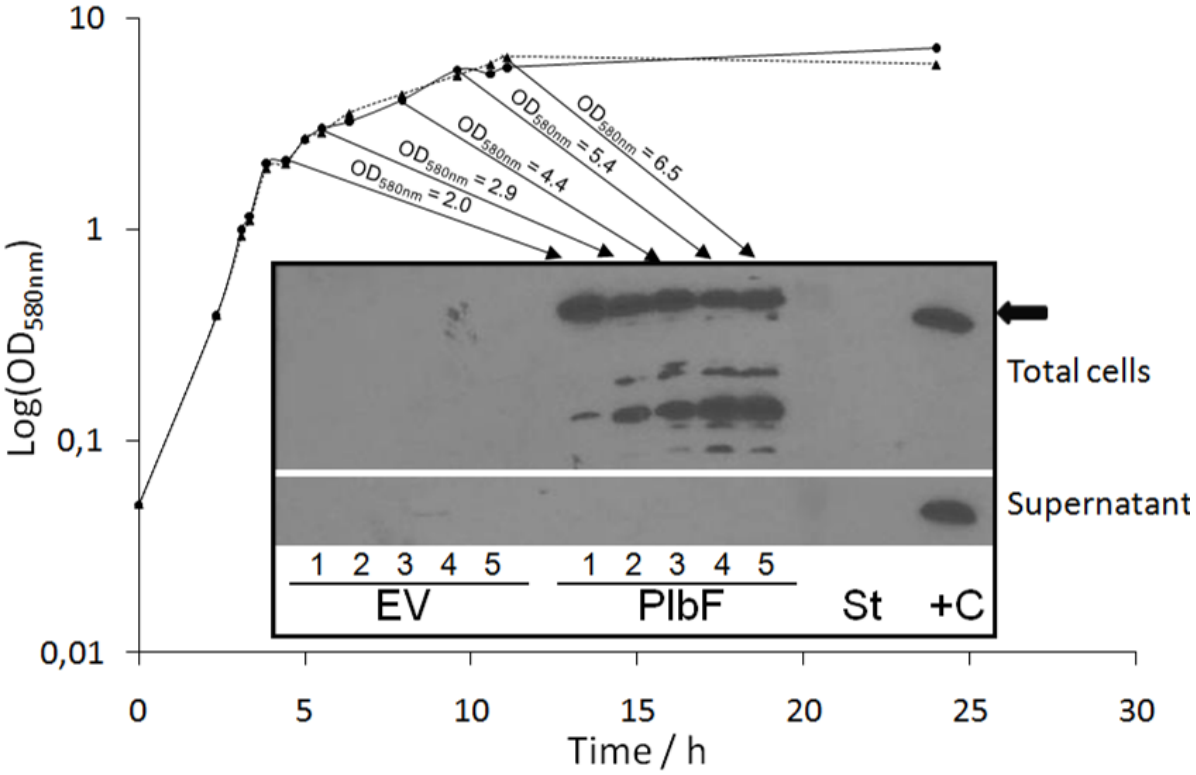


Figure 57: Localisation of PlbF as a cell-associated protein in *P. aeruginosa* PA01. Growth of *P. aeruginosa* PA01 transformed with pBBR1mcs-3 (EV = Empty vector, dashed line) and pBBR-PlbFH6 (PlbF, solid line) in TSB medium was compared under standard conditions. In the middle of the figure are Western blots with detection of PlbFH6 in total cell extracts (0.1 U_{Cell}/lane) and supernatants (1

U_{Cell}/lane) using anti-His6-tag antibodies (Chapter 3.18.4). St = molecular weight standards. A sample containing PlbFH6 with previously demonstrated immunoreactivity in Western blot was used as the positive control (+C). Cultures were examined at five growth points (1, 2, 3, 4 and 5) and respective OD_{580nm} values are indicated. Black arrow indicates PlbF.

In all following localisation experiments, we have analysed early growth phase cultures (corresponding to the first analysed point in Figure 57) for the following reasons: a) PlbFH6 is expressed in sufficient amounts for immunodetection; and b) degradation of PlbFH6 is weak at that growth point. Furthermore, the growth conditions in the following localisation experiments were identical to those in the experiment presented above.

The next step was to determine whether PlbFH6 is indeed a membrane-associated protein. Therefore, the *P. aeruginosa* PA01 cultures transformed with pBBR-PlbFH6 and pBBR1mcs-3 were disrupted by means of a French press and the unbroken cells were removed by a slow centrifugation step. Total membranes (inner and outer membranes) separated from soluble proteins using ultracentrifugation (Chapter 3.14.2) were analysed on SDS PAGE and Western blot (Figure 58A).

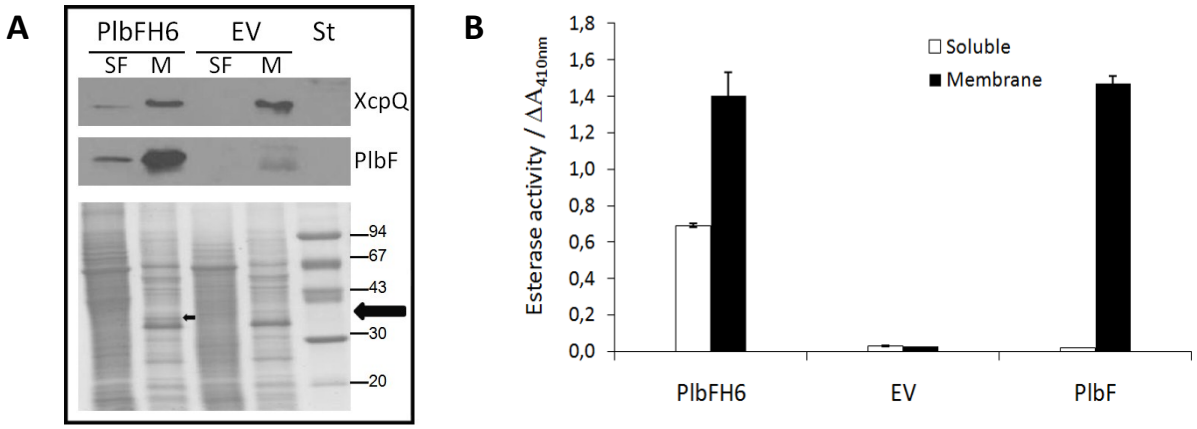


Figure 58: Subcellular localization of PlbF in membranes of *P. aeruginosa* PA01. **A)** The membrane (M) and soluble fractions (SF) of *P. aeruginosa* PA01 transformed with pBBR-PlbFH6 (PlbFH6) and pBBR1mcs-3 (empty vector, EV) were separated (Chapter 3.14.2) and the equivalent of 0.4 U_{Cell} for each cell compartment were analysed by Western blot (two upper panels) and SDS PAGE (lower panel). *P. aeruginosa* PA01 membrane protein (XcpQ) and PlbFH6 were immunodetected with anti-XcpQ and anti-His6-tag antibodies, respectively, on Western blots. The samples analysed by Western blot were loaded on an SDS PAGE gel. Molecular weights of protein standard (St) in kDa are indicated on the right. The black arrow indicates PlbFH6. **B)** Esterase activity assay for membrane and soluble fractions of *P. aeruginosa* PA01 transformed with pBBR1mcs-3 (EV), pBBR-PlbFH6 (PlbFH6) and pBBR-PlbF (PlbF). The esterase activity assay was performed with 2.5 µl (0.008 U_{Cell}) sample per 250 µl substrate solution, and the increase in A_{410nm} was continuously recorded during 45 sec. The results are means of three measurements.

Immunodetection of PlbFH6 with anti-His6-tag antibodies reveals a strong signal of PlbFH6 in the membrane fraction and a weak signal in the soluble fraction. To judge the purity of fractions, a membrane protein (XcpQ) was immunodetected. The results indicate a strong signal for XcpQ in the membrane fraction but a weak signal in the soluble fraction (PlbFH6-expressing strain). This suggests minor contamination of the soluble fraction with membranes, which could explain the detection of PlbFH6 in the soluble fraction. However, the intensities of the XcpQ and PlbFH6 signals are much stronger in the membrane than the soluble fraction; therefore, we can assume a membrane

localisation for PlbFH6. Moreover, standard SDS PAGE analysis revealed a ca. 35 kDa protein, which matches the theoretical molecular weight for PlbFH6 of 35.7 kDa, in the membrane fraction of the PlbFH6-expressing strain but not the empty vector strain (Figure 58A). This means that the amount of expressed PlbFH6 in the membrane fraction was sufficient to allow for its efficient visualization by standard SDS PAGE analysis.

To strengthen the data obtained by Western blot and SDS PAGE analyses, the esterase activities of membrane and soluble fractions from *P. aeruginosa* PA01 expressing PlbFH6 and PlbF were compared with the empty vector control. The results clearly indicate increasing esterase activity in membrane fractions of PlbF- and PlbFH6-expressing strains compared to the empty vector strain (Figure 58B). This observation confirms a membrane localisation for PlbF, which is in accordance with previously obtained Western blot and Ni-NTA purification results. The esterase activities for soluble fractions of the PlbF-expressing strain and empty vector control are roughly the same and very low. Interestingly, the esterase activity for the soluble fraction of the PlbFH6-expressing strain is much higher when compared to the PlbF-expressing and empty vector strains. The soluble fraction of the PlbFH6-expressing strain contains approximately 50% of the activity from membrane fractions of the respective strain. The observed difference between PlbF and PlbFH6 expressing strains indicates an influence of the His6-tag on the cellular localization of PlbF. It may be that the carboxy-terminal His6-tag causes a defect during insertion of PlbF into the membrane, which has consequences for mislocalization of PlbFH6 as a soluble protein. The results obtained here may explain the detection of soluble PlbFH6 by Western blot (Figure 58A). Furthermore, the same activities of membrane fractions from PlbF- and PlbFH6-expressing strains were observed, which suggests that the membrane insertion of PlbF is a limiting factor for expression of this protein.

The membrane localisation of PlbF in *P. aeruginosa* PA01 prompted us to determine whether PlbF is localised in the inner or outer membrane of this *gram-negative* bacterium. To answer this question, we separated inner and outer membrane proteins under native conditions using sucrose gradient ultracentrifugation. Isolation of total cell membranes (inner and outer membranes) from *P. aeruginosa* PA01 transformed with pBBR-PlbFH6 and pBBR1mcs-3 was performed as previously described (Chapter 3.14.2) and sucrose density gradient fractionation was described in detail in the Methods section (Chapter 3.14.3). The analysis of sucrose gradient fractionation was performed by Western blot detection of an inner membrane protein marker (XcpZ) [307] and outer membrane protein marker (XcpQ) [180] followed by the detection of PlbFH6 with anti-His6-tag antibodies (Figure 59A). XcpQ, representing outer membranes, was detected in fractions 10–18 (in empty vector and PlbFH6-expressing strains). Results of detecting the inner membrane protein XcpZ show that it extends from fraction 4 to fraction 18. PlbFH6 was detected in fractions ranging from 4 to 18. It is obvious that the Western blot profile of PlbFH6 matches the profile of XcpZ but not the profile of XcpQ. This result shows co-localization of PlbFH6 with inner membranes, and, therefore, it is most likely an inner membrane protein.

Using densitometric analysis of the Western blot fractionation profiles by AIDA software, the intensity of each signal was quantified (data not shown). According to these data the most intense signals for XcpQ were detected in fractions 15 and 16. Due to the low quality of XcpZ detection caused by poor and non-specific antibodies against the XcpZ protein, it was difficult to quantify the signal intensities accurately. However, the results reveal the strongest intensities for the XcpZ signals

in fractions 10 and 11. Based on these results, we consider fractions 15 and 16 as outer membranes and fractions 10 and 11 as inner membranes. However, from Western blot analysis it is obvious that fractions 10 and 11 do not exclusively contain inner membranes and fractions 15 and 16 do not exclusively contain outer membranes. Therefore, we have calculated the ratio between inner (sum of signal intensities of fractions 10 and 11) and outer (sum of signal intensities of fractions 15 and 16) membranes ($R_{Inn/Out}$) for XcpQ and XcpZ. The $R_{Inn/Out}$ values for XcpZ and XcpQ are 2.5 and 0.2, respectively. Analysing the PlbFH6 signals, an $R_{Inn/Out}$ value of 2.8 was calculated. These data reveal that almost three-fold (2.8) more PlbFH6 was detected in fractions 10 and 11 than in fractions 15 and 16, which is in good agreement with XcpZ (2.5 fold more) but opposite to XcpQ (five-fold less).

Taken together, the co-localisation of PlbFH6 with XcpZ (inner membrane protein), but not XcpQ (outer membrane protein), and the inner and outer membrane ratio for PlbFH6 ($R_{Inn/Out}$ = 2.8) that is similar to that for XcpZ ($R_{Inn/Out}$ = 2.5) but very different from that for XcpQ ($R_{Inn/Out}$ = 0.2), we can conclude that PlbFH6 is an inner membrane-associated protein of *P. aeruginosa* PA01.

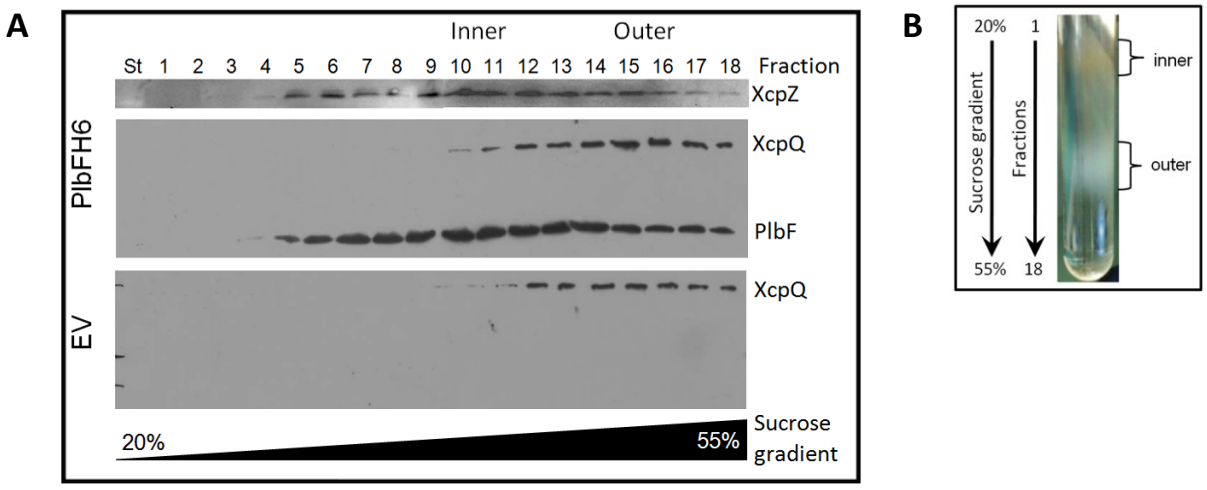


Figure 59: Localisation of PlbF in inner and outer membranes of *P. aeruginosa* PA01. **A)** Western blots of membrane fractions of *P. aeruginosa* PA01 transformed with pBBR-PlbFH6 (PlbFH6, two uppers panels) and pBBR1mcs-3 (EV, lower panel) in a sucrose gradient (Chapter 3.14.3). In total, 18 fractions of the gradient ranging from 20% (v/v) to 55% (v/v) sucrose were collected. Inner membrane protein (XcpZ), outer membrane protein (XcpQ) and PlbFH6 were detected on the Western blots with anti-XcpZ, anti-XcpQ and anti-His6-tag antibodies, respectively. **B)** Visualisation of a tube after separation of inner (white) and outer (brownish) membranes in the sucrose gradient. Separation of membranes isolated from *P. aeruginosa* PA01 expressing PlbFH6 is shown in the photograph.

To distinguish further between the peripheral and integral nature of membrane association for PlbF, *P. aeruginosa* PA01 membranes were treated with an alkali solution of sodium carbonate and the denaturating reagent urea. Both reagents destabilise weak protein-membrane interactions present in peripherally-associated proteins, resulting in their dissociation from membranes. The dissociation of integral proteins is not induced upon treatment with either sodium carbonate or urea. Triton X-100 non-specifically solubilises cell membranes, resulting in the release of both peripheral and integral proteins from membranes. Buffer lacking TX-100 and urea was used as a negative control. PlbFH6 was expressed in *P. aeruginosa* PA01, and membranes were isolated as in the previous experiments. After membrane treatment with sodium carbonate, soluble and insoluble proteins

following urea and TX-100 treatment were separated by centrifugation and analysed by SDS PAGE and Western blot.

Western blot detection of PlbFH6 with anti-His6-tag antibodies (Figure 60) revealed a PlbFH6 signal at the size of ca. 35 kDa in the insoluble fraction of membranes treated with sodium carbonate, urea and buffer, but signal was not detected in the respective soluble fractions. After treatment with TX-100, a PlbFH6 signal at the size of 35 kDa was detected only in the soluble fraction. The respective SDS PAGE analysis (Figure 60) revealed partial solubilisation of membrane proteins by sodium carbonate, urea and buffer, but complete solubilisation was achieved after treatment with TX-100 (2% (v/v)). The data presented demonstrate the resistance of membrane-associated PlbFH6 to solubilisation with sodium carbonate and urea and its sensitivity to solubilisation with TX-100. Therefore, we can conclude that PlbF is an integral membrane protein and not a peripheral membrane protein of *P. aeruginosa* PA01.

An anomaly in the Western blot analysis was the presence of signals for PlbFH6 at the size of ca. 70 kDa in insoluble membrane fractions treated with sodium carbonate and urea. The size of this signal corresponds to a dimeric form of PlbFH6. Furthermore, this signal was not observed in PlbFH6 membranes treated with buffer and TX-100. Therefore, we can conclude that these signals are exclusively a result of interactions of sodium carbonate and urea with PlbFH6. It is well known that alkali solution and urea cause dramatic changes in the tertiary structure of enzymes, resulting in their denaturation. It may be that two PlbFH6 molecules close to each other in the membrane interact upon denaturation, forming SDS-resistant dimers (Figure 61). Interestingly, Western blot signals for trimeric or multimeric PlbFH6 forms were not observed. It appears that this observed dimerisation and lack of multimerisation could be due to a possible dimeric organisation of PlbFH6 molecules in membranes under native conditions rather than due to interactions of PlbFH6 momomers inserted randomly in the membranes.

These results indicate that PlbF may be a homodimeric integral membrane protein in *P. aeruginosa* PA01 (Figure 60). The physiological meaning of PlbF dimerisation under native conditions cannot be completely understood at this point, but it certainly indicates that PlbF is an interesting membrane hydrolase of *P. aeruginosa* PA01.

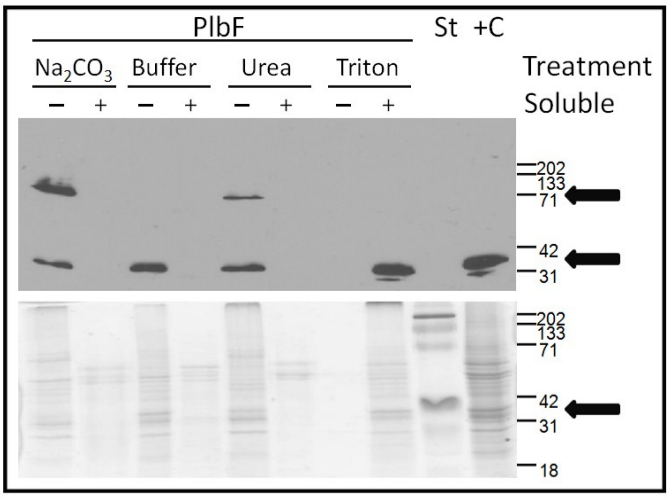


Figure 60: Integral membrane association of PlbFH6 in *P. aeruginosa* PA01. Western blot (upper panel) and Coomassie-stained SDS PAGE gel (14%) (lower panel) of membrane fractions (180 U_{Cell}/ml) from *P. aeruginosa* PA01 expressing PlbFH6 that were treated for 30 min at room temperature with sodium carbonate (Na_2CO_3, 50 mM), urea (4 M), Triton X-100 (2% w/v) and buffer (MES, 40 mM, pH 6.5) as a negative control. After the treatment, soluble (+) and insoluble (-) proteins were separated by ultracentrifugation. For both analyses, the equivalent of 0.09 U_{Cell} of each sample was loaded on the gel. PlbFH6 was immunodetected with anti-His6-tag antibodies. Molecular weights of protein standard (St) in kDa are indicated on the right. A sample containing PlbFH6 with previously demonstrated immunoreactivity in Western blot was used as the positive control (+C). Black arrows indicate monomeric and dimeric forms of PlbFH6.

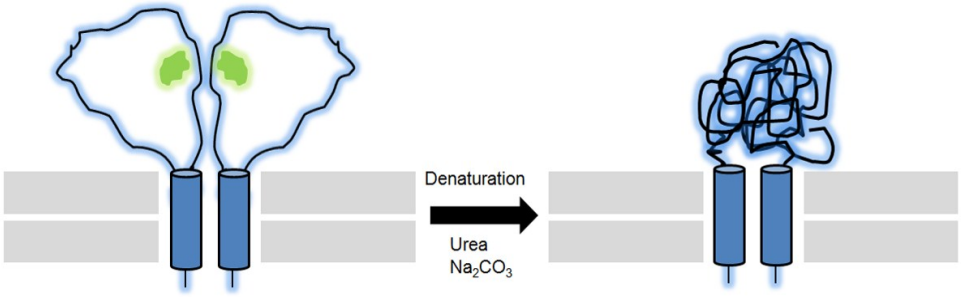


Figure 61: Simple model proposing denaturation-induced dimerisation of PlbF in *P. aeruginosa* PA01. Two membrane-anchored molecules of PlbF in close proximity interact with their C-terminal domains upon denaturation, forming a strong dimeric complex. Putative N-terminal transmembrane helices (blue cylinders) of PlbF are inserted in the membrane bilayer (gray areas) as represented, and C-terminal domains responsible for catalytic activity (green areas represents active sites) that are located out of the membrane are probably in close proximity.

To find out whether PlbF requires interaction with any *P. aeruginosa* PA01 protein for proper localisation in the membrane, PlbFH6 was localised in *E. coli*. The localisation experiments in *E. coli* were designed similarly to those in previous localisation experiments for *P. aeruginosa* PA01. Therefore, TSB medium was chosen for expression, cultures in the early logarithmic phase were analysed and the same plasmids (pBBR-PlbFH6 and pBBR1mcs-3) were used. Expression was performed in *E. coli* DH5α, and membranes and soluble proteins were separated as before. The detection of PlbFH6 with anti-His6-tag antibodies revealed signal in the membrane fraction of the strain expressing PlbFH6 (Figure 62). The intensity of the PlbFH6 signal on Western blots was weak,

indicating lower expression levels of PlbFH6 in *E. coli* DH5α than in *P. aeruginosa* PA01. Furthermore, the amount of PlbFH6 was insufficient for useful visualisation on an SDS PAGE gel, which is in keeping with the Western blot results indicating weak expression of PlbFH6. This result indicates a membrane association for PlbFH6 in a heterologous host, the same as was observed in a homologous host. Therefore, we can exclude the need for specific *P. aeruginosa* PA01 protein in the association of PlbF with membranes in *P. aeruginosa* PA01.

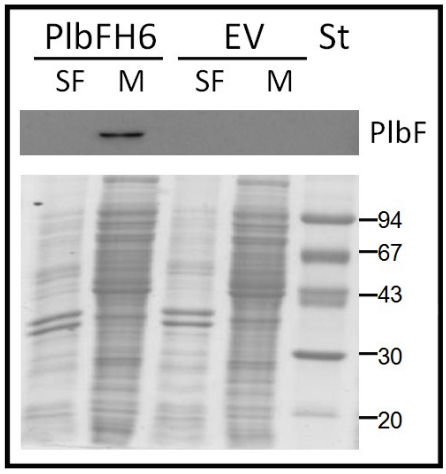


Figure 62: Subcellular localization of PlbF in membranes of *E. coli* DH5α. The membranes (M) and soluble fraction (SF) of *E. coli* DH5α cultures transformed with pBBR-PlbFH6 (PlbFH6) and pBBR1mcs-3 (empty vector, EV) were separated and 0.4 U_{Cell} of each cell compartment were analysed on Western blot (PlbF, upper panel) and subsequently on an SDS PAGE gel (lower panel). Molecular weights of protein standard (St) in kDa are indicated on the right. PlbFH6 was immunodetected with anti-His6-tag antibodies.

4.4.3.5. PlbF is a potential virulence factor of *P. aeruginosa* PA01.

For more than 20 years, the fruit fly *Drosophila melanogaster* has been widely used as a non-mammalian host organism for studying bacterial infections [308]. The signalling pathways and tissue physiology are conserved between *D. melanogaster* and mammals, allowing modelling of human host-pathogen interactions in *D. melanogaster* [183]. Many examples using this model host organism exist for the identification of virulence factors [309, 310, 311] and for the elucidation of virulence mechanisms of *P. aeruginosa* [308]. Recently, it has been published that the needle-pricking method can be used for infection of *D. melanogaster* with *P. aeruginosa*, which may be employed for the identification of specific genes responsible for bacterial infection [183].

The profiles of *D. melanogaster* responses to *P. aeruginosa* PA01 (wild type) and a *P. aeruginosa* strain lacking the *plbF* gene (mutant) were determined using the needle-pricking method (Chapter 3.22). Flies pricked with the wild type strain died more quickly than those pricked with the *plbF* mutant strain (Figure 63). Forty-five h after pricking with wild type, ca. 20% of flies survived, compared with ca. 80% of flies after pricking with the *plbF* mutant strain. Therefore, the observed attenuation for the *plbF* mutant in killing *D. melanogaster* is likely linked to the lack of *plbF* in this strain. Although, we have observed, under different conditions than in vivo in *D. melanogaster*, that the *plbF* mutant strain is not impaired in its growth (LB medium, 37˚C) when compared to the parental strain (data not shown). It is not possible to state whether PlbF itself has influence on *D. melanogaster* or it beneficially contribute to the *P. aeruginosa* physiology and therefore influence virulence. However, these data indicate that PlbF is a novel virulence factor for *P. aeruginosa* PA01. Moreover, it is the first potential virulence factor of *P. aeruginosa* PA01 belonging to the phospholipase A2 group.

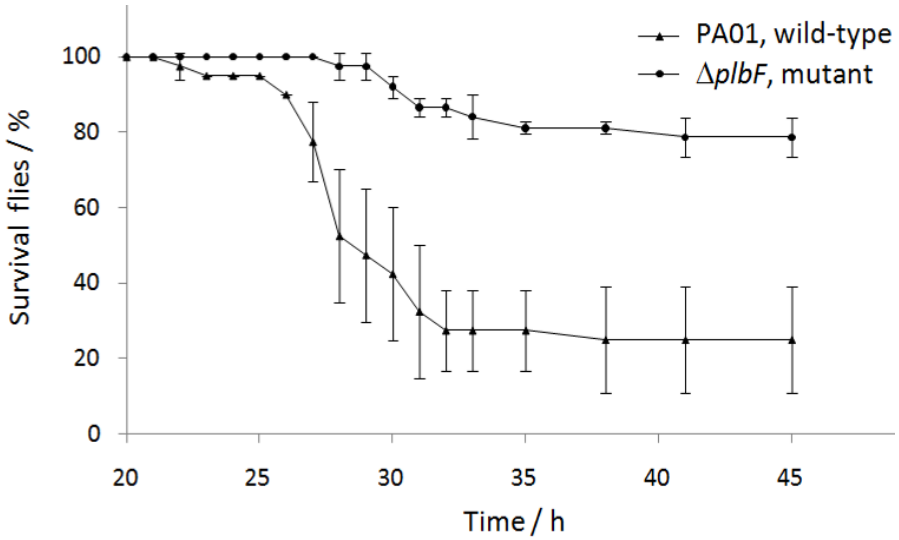


Figure 63: Killing of *D. melanogaster* with *P. aeruginosa* PA01 (PA01, wild type) and a strain mutant for *plbF* (Δ*plbF,* mutant). Twenty flies were locally infected with 100 bacteria from wild type and mutant strains grown in LB medium until stationary phase (OD_{580nm} = 3). Live flies were counted over a period of 45 h. The results are means of three experiments.

Summary

4.4.3 Membrane bound phospholipase B, PlbF of *P. aeruginosa* PA01

PlbF displays sequence homology to lipases, esterases and phospholipases from prokaryotes and eukaryotes (Table 13). Putative or demonstrated functions of these homologues (lipid metabolism and lipid signalling) are of great importance for the homologous host. Therefore, it is interesting to demonstrate the enzymatic activity of PlbF experimentally to relate it to those physiologically important classes of enzymes.

A multiple sequence alignment of PlbF with α/β-hydrolases whose active sites were inferred from three dimensional structures (Figure 19) indicates Ser137, Asp286 and His258 as a putative catalytic triad of PlbF.

Heterologous expression of PlbF in *E. coli* BL21(DE3) under the control of P_{T7} did not yield sufficient amounts of PlbF for visualisation by standard SDS PAGE analysis. Nevertheless, cells expressing PlbF exhibit high levels of esterase activity that were not observed in the empty vector control (Figure 40). Furthermore, *P. aeruginosa* PA01, *E. coli* DH5α and *E. coli* BL21(DE3) cells expressing PlbFH6 were active in an esterase activity assay, in contrast to the respective strains transformed with empty vector (Figures 40 and 58B). The PlbFH6 expressed in all three above mentioned strains was furthermore immunodetected as a ca. 35 kDa protein (Figures 58A and 62). These results confirmed that PlbF and PlbFH6 were expressed in *E. coli* and *P. aeruginosa* as functional esterases.

E. coli BL21(DE3) cells expressing PlbF displayed significantly more activity against PLA1, PLA2 and thioesterase substrates compared to the empty vector control cells (Figure 53). Furthermore, the esterase activity of the same cells was inhibited by the serine hydrolase inhibitors PMSF (100% inhibition) and THL (60% inhibition) (Figure 54). Therefore, we have concluded that PlbF is an esterase, PLA1, PLA2 and thioesterase that is very sensitive to PMSF and moderately sensitive to THL.

In later stages of this study, PlbFH6 was expressed in *P. aeruginosa* PA01, solubilised from membranes and purified on a Ni-NTA column in the presence of Triton X-100. Pure PlbFH6 exhibits esterase activity, confirming our previous findings with total lysates from cells expressing PlbF and PlbFH6. However, difficulties in purification of PlbFH6 and instability of pure PlbFH6 are reasons why pure PlbFH6 was not subjected to further biochemical studies.

In silico protein localization methods suggest a signal peptide for PlbF (Table 3); therefore, it was predicted to be a putative extracytoplasmic protein. Experimentally, we have demonstrated that PlbFH6 is expressed in *P. aeruginosa* PA01 as a cell-bound protein and not as an extracellular protein (Figure 57). Additionally, the same experiment shows that PlbFH6 is expressed already at the early growth phase and its degradation increases upon culture growth. Furthermore, Western blot and SDS PAGE analyses of total membranes (inner and outer) and soluble proteins (periplasmic and cytoplasmic) from *P. aeruginosa* PA01 expressing PlbFH6 revealed membrane localisation of PlbFH6 (Figure 58A). The esterase activities of *P. aeruginosa* PA01 expressing PlbFH6 and PlbF are also membrane-associated (Figure 58B). However, the esterase activity present in the soluble fraction of *P. aeruginosa* PA01 expressing PlbFH6 but absent in the strain expressing PlbF or empty vector strain indicates the influence of the carboxy-terminal His6-tag on mislocalisation of PlbFH6.

To find out which membrane binds PlbF, the inner and outer membranes of *P. aeruginosa* PA01 expressing PlbFH6 were separated in a sucrose density gradient. Subsequent Western blot analyses revealed co-localization of PlbFH6 with an inner membrane protein (XcpZ) rather than an outer membrane protein (XcpQ) (Figure 59). Furthermore, the resistance of PlbFH6 to solubilisation with urea and sodium carbonate implicates that PlbF is an integral and not a peripheral membrane protein (Figure 60).

The confirmed membrane localisation of catalytically active PlbFH6 expressed in *E. coli* indicates that PlbFH6 does not require specific *P. aeruginosa* protein for its proper function and localisation (Figure 62).

To study the relationship between PlbF and *P. aeruginosa* PA01 virulence, a strain lacking *plbF* (Δ*plbF*) was constructed and subsequently compared to the parental strain (PA01) in a *D. melanogaster* virulence model. Attenuation of the Δ*plbF* strain in the killing of flies compared to the wild type strain was observed (Figure 63). These data point to PlbF as a novel virulence factor of *P. aeruginosa* PA01, making it the first phospholipase A2 with a potential contribution to the virulence of *P. aeruginosa* PA01.

5. Discussion

5.1. General discussion

Phospholipases are ubiquitous and diverse enzymes that have in common the substrate which they hydrolyse, a phospholipid. They are classified into four major groups (A, B, C and D) (Figure 1) based on their specificity to cleave different ester bonds in phospholipids. From a physiological point of view, the four phospholipase groups are interrelated and, together with lipases, phosphatases, acyltransferases and lipid kinases, provide a vast array of lipid mediators involved in a variety of **eukaryotic cellular processes** (Figure 64). Nevertheless, depending on the production of different bioactive lipid mediators that regulate several physiological processes, phospholipases may be classified into three physiologically distinct classes: PLC, PLD and PLA2. The most commonly described phospholipases are the PLC enzymes, while less is known about the PLD group. The PLA2 class of phospholipases is the largest group of phospholipases, with a body of literature comparable to that of PLC enzymes. Some of phospholipases functions in eukaryotes are as follows:

a) PLC enzymes hydrolyse phospholipids to generate two lipid mediators, diacylglycerol (DAG) and inositoltriphosphate (IP3), which activate protein kinase C and release intracellular calcium, respectively. This is known as the PLC signalling pathway, which is involved in regulating cell proliferation and differentiation, endocytosis, chemotaxis and apoptosis [312, 313].

b) PLD enzymes hydrolyse phospholipids to phosphatidic acid (PA) and free choline (Ch). While choline does not perform any signalling role, PA has been linked to vesicular trafficking, secretion, prevention of apoptosis and endocytosis through the activation of PI(4)P 5-kinase, Raf-1 kinase and mTor, which are effector molecules involved in cell cycle progression and cell proliferation.

c) PLA2 enzymes cleave the ester bond on the sn2 position in phospholipids, allowing for the release of two biologically active lipid molecules: fatty acid and lysophospholipid. However, PLA2 enzymes are characterised by the ability to carry out multiple enzymatic reactions, including those also mediated by PLA1, PLB, LysoPLA, lipase/esterase and transacylase [55]. Therefore, the classification of enzymes in the PLA2 class is rather difficult. The physiological importance of PLA2 is the fact that the most common fatty acid linked to the sn2 position of eukaryotic phospholipids is arachidonic acid (AA), which is the most physiologically relevant fatty acid [43, 314]. Arachidonic acid is the precursor to a large family of proinflammatory eicosanoids that includes prostanoids, lipoxines and leukotrienes [96]. The cellular pathways controlled by these lipid mediators regulate a variety of physiological and pathophysiological processes. Several examples include the following: ovulation; proper functioning of the respiratory tract and defects in the functioning of the respiratory tract that result in bronchial asthma [314]; and regulation of innate and acquired immune responses [93] that can promote cystic fibrosis and atherosclerosis [314]. The second molecule generated by the breakdown of phospholipids by PLA2 is lysophospholipid, a precursor of platelet-activating factor (PAF). PAF has been implicated both in haemostasis and in pathophysiological conditions, such as allergic asthma, endotoxin shock, acute pancreatitis and dermal inflammation [94].

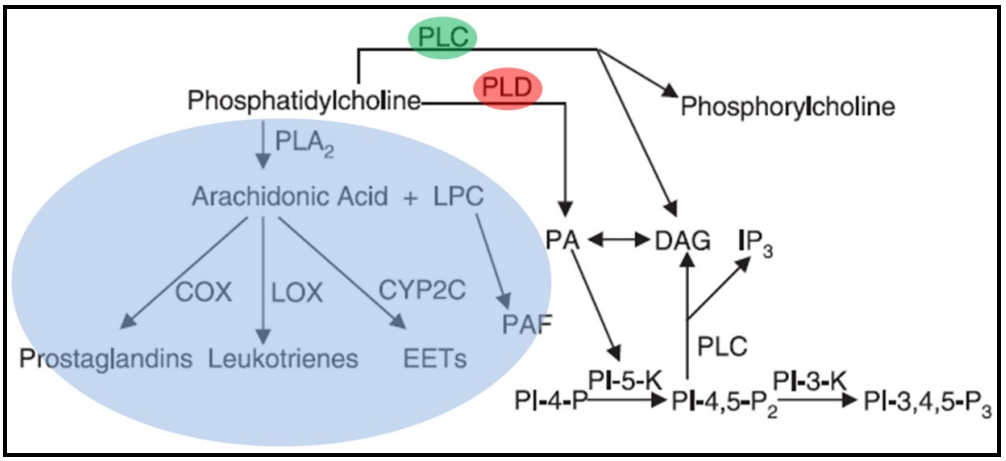


Figure 64: The interrelatedness of phospholipase A2 (PLA2), phospholipase C (PLC) and phospholipase D (PLD) and interconvertibility of the lipid signalling pathways. LPC, lysophosphatidylcholine; COX, cyclooxygenase; LOX, lipoxygenase; CYP2C, cytochrome P-450 2C; EETs, eicosatrienoic acids; PAF, platelet-activating factor; PA, phosphatidic acid; DAG, diacylglycerol; IP_3, inositol (1,4,5)-trisphosphate; PI(4)P, phosphatidylinositol (4)-phosphate; $PI(4,5)P_2$, phosphatidylinositol (4,5)-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol (3,4,5)-trisphosphate; PI5K, phosphatidylinositol 5-kinase; PI-3-K, phosphatidylinositol 3-kinase. Modified figure from Eyster (2007) [314].

The data presented here reveal that products of phospholipase activity, in particular arachidonic acid, act as potential points for the interaction of pathogens with the host to initiate an inflammatory response that could lead to the tissue necrosis typically observed in *P. aeruginosa* infections. Indeed, the immunomodulatory capacity of some bacterial PLA2 enzymes was demonstrated and described in detail for *P. aeruginosa* ExoU, *Yersinia enterocolitica* YplA and group A *Streptococcus* SlaA. The ExoU is recognised as an important cytotoxin and immunomodulator that triggers the arachidonic acid cascade *in vivo*, thus inducing inflammation [114]. However, it is apparent that aside from the direct harmful effects (cytotoxic and proinflammatory) to the host, PLA2 enzymes facilitate bacterial colonisation (ExoU) and adherence (SlaA, *E. coli* OMPLA [82]) and therefore indirectly contribute to the virulence of PLA2-expressing strains [43]. In light of the multiple molecular mechanisms (toxic, signal modulation and adherence) by which the PLA2 class of enzymes contribute to bacterial virulence, it is likely that these enzymes represent attractive targets to combat bacteria-mediated disease.

In conclusion, it should be mentioned that, although there is clear evidence about the involvement of PLA2 in bacterial pathogenesis, the lack of purified PLA2 enzymes from pathogenic or non-pathogenic bacteria make it difficult to determine their precise function in pathogen-host interactions.

5.2. PLA2 and LysoPLA activities of *P. aeruginosa* PA01

Because the PLA2 enzyme in *P. aeruginosa* PA01, a model organism for investigation of pathogen-host interactions, was not previously described, herein we addressed whether this organism had PLA2 activity and determined where PLA2 localised within the bacterial cell. As discussed above, PLA2 molecules are often multifunctional enzymes that may possess LysoPLA activity, among others. It was previously demonstrated that *P. aeruginosa* ExoU [107] and *L. pneumophila* PlaA [123] have

both PLA2 and LysoPLA activity. However, the functions of PLA2 and LysoPLA could be different, as LysoPLA has been associated with the detoxification of lysophospholipids in bacteria [123]. Therefore, we have examined, in addition to PLA2 activity, the LysoPLA activity in *P. aeruginosa* PA01 cells.

We identified periplasmic and membrane-associated PLA2 and LysoPLA activities in *P. aeruginosa* PA01 (Figure 10A). The purity of the subcellular fractions was confirmed by immunodetection (Figure 10B), demonstrating that these two fractions were not contaminated with components from other cell compartments. Therefore, we can conclude that PLA2 and LysoPLA activities may be assigned exclusively to periplasmic and membrane protein(s). Furthermore, the levels of cytoplasmic and extracellular PLA2 and LysoPLA activities were below detection limit. Therefore, we assumed that under these growth conditions, *P. aeruginosa* PA01 had no mentionable cytoplasmic and extracellular PLA2 and LysoPLA activities. It is notable that 19% and 13% more PLA2 and LysoPLA activity, respectively, were measured in the periplasm compared to the membrane compartment (Figure 10A). Therefore *P. aeruginosa* PA01 most likely expresses different PLA2/LysoPLA (since many PLA2 enzymes have LysoPLA activity) enzymes that are periplasmic and membrane-associated. The identification of these enzymes and establishment of their relationship to *P. aeruginosa* virulence is our major aim.

Although most of the literature described extracellular PLA2 activity as a potential virulence factor, cell-associated PLA2 activities were also described as virulence factors in some human pathogens. The only human pathogen that was identified to have periplasmic and membrane PLA2/LysoPLA enzymes was *E. coli*, which produces outer membrane PLA/LysoPLA (OMPLA) [75], inner membrane LysoPLA [315] and periplasmic LysoPLA (TesA) [44]. Furthermore, membrane-associated haemolytic PLA2 was described for *Mycobacterium tuberculosis* (mycobacterial PLA [316]) and *Campylobacter coli* (outer membrane PLA/LysoPLA [317]). Moreover, cell-associated PLA and LysoPLA activity was reported for *L. pneumophila* [198]. The latter study demonstrated that one enzyme, PlaB, contributed almost exclusively to both of these activities. Another recent study of *L. pneumophila* PlaB suggested that PlaB localised to the outer membrane and played a role in the virulence of the bacteria in a guinea pig model [122].

It should be mentioned that there is the possibility of positive or negative regulation of PLA2 and LysoPLA activities during the infection, similar to the regulation of expression of many extracellular and cell-associated virulence factors of *P. aeruginosa* [8]. Thus, the secreted form of SlaA from *Streptococcus* was enhanced upon contact of bacteria with epithelia cells and human saliva [104].

5.2.1. Influence of cell density and media composition on PLA2 activity of *P. aeruginosa* PA01

Data regarding the induction of PLA2 activity in *P. aeruginosa* PA01 at different growth stages and in different growth media should provide a basic knowledge of this novel class of enzymes not previously described in this organism. Furthermore, such data may be helpful in the later stage of the study, which will focus on the identification of a particular gene or protein possessing PLA2 activity, to decide at which growth stage cultures should be examined and which media should be selected to promote high or low PLA2 activity.

We have observed that *P. aeruginosa* PA01 has cell-associated PLA2 activity at all growth stages; PLA2 activity is low in early growth stages (logarithmic), and it increases when entering the stationary phase (Figure 11). The requirement for bacterial PLA2 in housekeeping cellular processes, e.g. membrane remodelling [71], lipid metabolism [72], and nutrient utilisation [39], could explain the need for *P. aeruginosa* PA01 to constitutively express PLA2 during the growth. Outer membrane PLA (OMPLA), detected in many pathogenic and non-pathogenic *gram-negative* bacteria, is an example of one PLA that has proposed housekeeping function based on its tightly controlled activity by substrate inhibition, which is typical for housekeeping enzymes [76]. Furthermore, OMPLA has the ability to change the membrane composition of *H. pylori* by increasing the pH resistance of bacteria [83]. Moreover, discovery of housekeeping enzymes present at the surface of pathogenic bacteria suggests that these enzymes can interact with the host and thus contribute to bacterial virulence [318]. Indeed, there is evidence that glyceraldehyde-3-phosphate dehydrogenase (a glycolytic enzyme) from enteropathogenic *E. coli* is secreted when *E. coli* encounters epithelial cells and is essential for signal transduction in host cells that results in disease [319]. Another glycolytic housekeeping enzyme, enolase, from *Streptococcus pneumoniae*, was demonstrated to promote virulence, as mutant bacteria expressing enolase that was impaired in binding to the plasminogen were attenuated in a mouse model of infection [320]. Although some essential bacterial enzymes have proven to be or are putative virulence factors; there is no evidence that such a dual function exists for bacterial phospholipases. It is known that bacterial PLA2, like *P. aeruginosa* ExoU and *Streptococcus* SlaA, increases bacterial colonisation and has cytotoxic and proinflammatory effects [43]. Furthermore, the expression of virulence traits is metabolically costly and so bacteria may express such factors co-ordinately at the site for optimal colonisation in the host [14].

Based on this view, the PLA2 activity expressed continuously during bacterial growth is likely not related to the virulence of *P. aeruginosa* PA01 but instead reflects the participation of PLA2 in other cellular processes. However, the strong evidence supporting the involvement of PLA2 in bacterial pathogenesis still highlights this particular enzyme group as a potent virulence factor of *P. aeruginosa* PA01.

When we monitored the activity of PLA2 in *P. aeruginosa* PA01 in response to nutrient limitation, we observed diminished activity. We then compared the PLA2 activity in cells grown in LB, PPGAS or MME media and found the highest activity in cells grown in the LB medium. Cells grown in minimal medium (MME) and cells grown in complex phosphate limiting medium (PPGAS) demonstrated 38% and 50%, respectively, of PLA2 activity compared to cells grown in LB medium. *P. aeruginosa* is a microorganism that can live in water, soil, and humans and thus has a remarkable metabolic versatility. However, *in vitro* [321] and *in vivo* [39] studies of the metabolic requirements of *P. aeruginosa* for growth at high cell density suggests that amino acids are very important nutrient sources. The major energy source in LB media is peptides/amino acids (ca. 15 g/l), in PPGAS, the nutrients are peptides (10 g/l) and glucose (5 g/l) and in MME, energy comes solely from glucose (2 g/l). The 50% less PLA2 activity in PPGAS compared to LB media may be influenced by the higher concentration of preferable nutrients (peptides/amino acids) in LB. The phosphate limitation in PPGAS is most likely not the reason for reduced PLA2 activity because *P. aeruginosa* PA01 grown on minimal media (with glucose as the sole energy source) containing 10 mM, 50 mM or 100 mM of phosphate does not alter PLA2 activity (data not shown). Furthermore, the absence of any amino acid source in MME, and even when the amount of glucose is reduced to 2 g/l in MME, results in an

additional 25% reduction in PLA2 activity compared to PPGAS. Although the mechanism of the observed effects of nutrient content on the PLA2 activity of *P. aeruginosa* PA01 is not completely understood, it is doubtless that media rich in peptides/amino acids is favourable for PLA2 activity. Therefore, in future experiments, LB medium will be used as the standard media for assessing PLA2 activity.

5.2.2. Outer membrane esterase (EstA) of *P. aeruginosa* PA01 exhibits PLA2 activity

In our previous experiments, we described the PLA2 activity of *P. aeruginosa* PA01 that may not be related to any enzyme described in the literature. However, work by Gdynia [1] demonstrated the PLB activity of outer membrane esterase (EstA) from *P. aeruginosa* PA01 [28]. Therefore, we were interested in the contribution of EstA to the total PLA2 activity of *P. aeruginosa* PA01. To examine this, we compared the PLA2 activity of a *P. aeruginosa* PA01 strain deficient in the *estA* gene [1] to the parental strain.

Prior to this experiment, we quantitatively measured the PLA2 activity of pure EstA with the PLA2 substrate used in our measurements (dithio diheptanoyl phosphatidylcholine), which differs from the substrate (phosphatidylcholine isolated from egg yolk) used in the previous study of EstA [2]. EstA was expressed in *E. coli* BL21 (DE3) where it formed inactive aggregates that were removed from the soluble proteins and cellular membranes after cell disruption by slow centrifugation. This procedure yielded inactive EstA of sufficient purity for biochemical studies (Figure 13B). The activation of EstA required dilution of urea-solubilised inclusion bodies in buffer containing detergent (Triton X-100), which assists folding of the protein into its catalytic active form. It should be mentioned that some membrane proteins (*E. coli* OMPLA [322], *Rhodopseudomonas blastica* porin [323]) display low refolding yield *in vitro*; thus, refolding may result in a mixture of properly folded (active) and misfolded (inactive) enzymes. Although the efficiency of EstA refolding was not examined, we confirmed EstA reactivation by an esterase activity assay. The EstA enzyme, active as an esterase, was subjected to the PLA2 activity assay and demonstrated activity (0.16 U/μg) of refolded EstA against the substrate (Figure 13). After *in vitro* PLA2 activity of EstA was confirmed, we measured PLA2 activity in the *P. aeruginosa* PA01 strain lacking the *estA* gene and wild type *P. aeruginosa* PA01. After insertion of EstA into the outer membrane and translocation of its catalytic (esterase) domain (also called the passenger domain) across the outer membrane, EstA remains associated with the cell membrane [28]. This is different from other outer membrane proteins (*P. aeruginosa* PlpD lipase [247], *N. gonorrhoeae* IgA1 protease [324]), which are cleaved and their catalytic domains are released into the extracellular space. Knowing the membrane localisation of EstA, we next analysed membranes, soluble cellular proteins (in the periplasmic and cytoplasmic compartments) and total cell lysates of wild type *P. aeruginosa* and the *estA* mutant. As expected, deletion of the *estA* gene resulted in a reduction in PLA2 activity in total cell lysates and membranes by 13%. However, the PLA2 activity in the soluble fraction was the same between the *estA* negative mutant and the wild type strain. This data confirmed the *in vivo* PLA2 activity of EstA and the existence of another membrane bound, soluble PLA2 in *P. aeruginosa* PA01 that contributed to the PLA2 activity in the *estA* negative strain.

P. aeruginosa is known as an "enzyme factory" with very active protein secretion, which is perhaps a consequence of the ubiquitous lifestyle of this pathogen. To date, 8 lipolytic enzymes of *P. aeruginosa* PA01 have been discovered, including lipases, esterases, and phospholipase C and D enzymes (Table 1). However, phospholipase A1, A2 and B enzymes have not been described in *P. aeruginosa* PA01, although the function of these enzymes, particularly PLA2, as virulence factors has been suggested in the recent past. The first stage of our study of PLA2 enzymes from *P. aeruginosa* PA01 demonstrated that *P. aeruginosa* PA01 possesses lipolytic activities (PLA2 and LysoPLA), which have not been assigned to any enzyme so far. Our future objectives will be to ascribe PLA2 activity to the specific protein of *P. aeruginosa* PA01. To accomplish this, the *P. aeruginosa* PA01 genome will be explored using *in silico* methods to define a putative PLA2.

5.3. Bioinformatics analysis of putative PLA

The progress in genome sequencing achieved in last decade resulted in availability of hundreds of complete sequences of bacterial genomes and the publication of the entire sequence of the human genome, which is fundamental to the future of biomedical research and therapeutic medicine [325]. In the vast array of gene sequences, a large portion (30-50%) are genes of unknown function [199]. Furthermore, up to 15% of genes that have some homology to already known genes are not correctly annotated [199]. The function of 43% of the genes of *P. aeruginosa* PA01 remains unknown (Figure 65), and thus this organism fits into the fuzzy picture of functional genomics. It is surprising how many genes of *P. aeruginosa* are of unknown function considering that *P. aeruginosa* PA01 a) is an organism of particular clinical relevance, b) has emerged as the model organism for studies of host-pathogen interactions and c) is a model organism for biofilm studies. Due to the high percentage of genes with unknown function in genomes, high throughput studies reveal always 40–50% of hits in group of genes with unknown function. For example, in two microarray studies initiated to identify novel virulence factors [326] and the factors distinguishing the biofilm and planktonic lifestyles [327] of *P. aeruginosa*, 46% and 53% of the genes, respectively, were identified with unknown function. This emphasises the need to determine the function of the unknown genes in the *P. aeruginosa* PA01 genome. Therefore, we focused our research exclusively on genes that have not been studied previously.

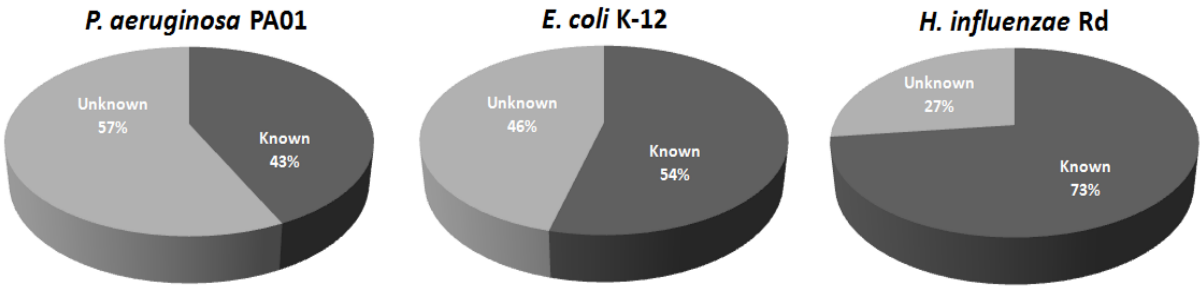


Figure 65: The distribution of experimentally characterised genes (Known) and genes without any experimental evidence of their function (Unknown) in *P. aeruginosa* PA01 [19], *E. coli* K-12 [328], the best studied bacterium, and *H. influenza* Rd, the first bacterium with an annotated genome [329].

5.3.1 Selection of putative phospholipase A enzymes from *P. aeruginosa* PA01

In our approach, putative PLA/PLB enzymes from *P. aeruginosa* PA01 were selected by screening the Pseudomonas Genome Database with the key words “phospholipase”, “esterase” and “lipase”. There are three reasons why esterases and lipases were included in the search. First, numerous esterases and lipases display lysophospholipase and phospholipase A1, A2 or B activity [55]. Second, the sequence features identified in esterases/lipases are similar to those found in phospholipases A. Third, the physiological interconnection between lipases/esterases and phospholipase A/B enzymes is possible because their substrates and products could have the same function in the cell. For example, potentially bioactive fatty acid could be released from phosphatidylcholine by PLA/PLB activity or from mono-, di- or triacylglycerol by lipase/esterase activity. The Pseudomonas Genome Database provides extensive genome annotation of sequence homology based on algorithms like BLAST, PFAM and others. Therefore, we preferred the text search to the sequence search, which resulted in 114 hits of lipolytic enzymes. This pool of enzymes was then manually screened for previously described enzymes (with existing primary literature), putative enzymes (with homology to known enzymes) and hypothetical enzymes (without homology to any enzyme). Ten previously described enzymes are specified in Table 1, but these enzymes are not our primary interest.

Out of the 114 lipolytic enzymes, 16 are putative and 88 are annotated as hypothetical enzymes. In the group of putative lipolytic enzymes (Table S1), seven were putative lipases, esterases and phospholipases, and all were included in the group of enzymes analysed herein. Among the hypothetical enzymes (Table S2), sequence homology suggested three potential PLA/PLB enzymes, PA4921, PA2927 and PA3339. Each of these potential enzymes displays homology to enzymes with demonstrated phospholipase A or B activity, which are the enzymes established as virulence factors in pathogenic bacteria. PA4921 is homologous to extracellular phospholipase A from *L. pneumophila* (PlaA) [123]; PA2927 is homologous to haemolytic phospholipase A from *L. pneumophila* (PlaB); and PA3339 is homologous to type III secreted exotoxin with phospholipase A activity from *P. aeruginosa* PA14 (ExoU). These three putative PLA/PLB enzymes together with the seven putative lipases, esterases and phospholipases (Table 12) mentioned above, were chosen for further study. The set of 10 putative PLA/PLB enzymes, which we named the “phospho-lipolytic system”, comprises four putative esterases (PA1047, PA2098, PA2856, PA3628), five putative phospholipases (PA2155, PA2927, PA3339, PA4339, PA4921) and one putative lipase (PA2949).

One candidate, PA1615, was not included even though it was annotated as a putative lipase with an identifiable GDSL-hydrolase motif. The reason is the absence of sequence features that are essential for the catalytic activity of GDSL-hydrolases. The detailed sequence analysis of PA1615 revealed poor homology to known GDSL-hydrolases, and the strongly conserved DXXH motif, which comprises aspartate and histidine of the catalytic triad, is not present in its sequence. Moreover, other residues downstream of the GDSL-motif that could contribute to the catalytic triad of the putative GDSL-lipase were not detected. The second notable difference between PA1615 and typical GDSL-hydrolases is the location of the GDSL-motif in the middle of the PA1615 sequence, when typically, the GDSL-motif is located exclusively in the first ca. 30 amino acids. The third difference between PA1615 and other GDSL-hydrolases is the protein size. PA1615 is a relatively small protein of 113 amino acids, while the other GDSL-hydrolases and hydrolases in general have a size of 200 or more amino acids. Taken together, we conclude that PA1615 is not a typical GDSL-hydrolase and we have not included it to our phospho-lipolytic system.

Extensive analysis of the 87 hypothetical lipolytic enzymes detected in *P. aeruginosa* PA01 identified an additional two GDSL-hydrolases. The open reading frames of PA3125 and PA3750 encode for two hypothetical proteins that have a GDSL-motif. Sequence alignment of these two enzymes with three previously mentioned GDSL-hydrolases from *P. aeruginosa* PA01 (EstA, TesA and PA4921) revealed conservation of all four sequences characteristic of GDSL-hydrolases (data not shown). However, the two enzymes were not homologous to any phospholipase, lipase or esterase, and so they were not included in our group of phospho-lipolytic enzymes.

Sequences of 10 phospho-lipolytic enzymes were analysed in silico to determine the functional homology, subcellular localisation and molecular organisation of phospho-lipolytic enzymes. Based on the conservation of essential catalytic motifs (active sites) in phospho-lipolytic enzymes and previously described enzymes, hints about the possible functions of phospho-lipolytic enzymes may be obtained. Furthermore, prediction of sequence features, such as signal peptides, transmembrane helices and outer membrane protein elements, give indications about the subcellular localisation of the enzymes. Thus, a functional model (Figure 26) and a localisation model (Figure 27) to predict the enzymatic function and subcellular localisation of phospho-lipolytic enzymes, respectively, were established. These two models were used as a starting point in the design of an experimental approach to analyse the novel lipolytic enzymes in *P. aeruginosa* PA01.

Each member of the phospho-lipolytic system was screened against a database of enzymes in which the three-dimensional structures are deposited (PDB), and against the manually annotated and reviewed section (Swiss-Prot) of the central protein sequence database, UniProt Knowledgebase. The proteins included in the two databases often have described active sites and enzymatic functions. Therefore, the homologues found in the databases serve as a reliable prediction of enzymatic function and subcellular localisation.

The sequence analyses of PlaK, TesA and PlbF will be extensively discussed in the last chapter, which is dedicated specifically to each enzyme. Another seven members will be briefly discussed jointly. However, it is useful to mention that at this point, we have named each phospho-lipolytic enzyme based on the sequence homology or experimentally determined enzymatic function. These names are not available in the Pseudomonas Genome Database, but table 12 gives their unique “PA numbers”, which allows for unambiguous identification of each enzyme to a certain gene of *P. aeruginosa* PA01. The only exception is the TesA protein (PA2856), which is published under this name in the Pseudomonas Genome Database.

According to the functional homology model, phospho-lipolytic enzymes may be classified into three groups: **a)** enzymes with homology to phospholipase D enzymes, **b)** enzymes with homology to esterases and lipases but without homology to phospholipase A/B enzymes, and **c)** enzymes with homology to phospholipase A/B enzymes. Based on our objectives (identification of phospholipase A enzymes of *P. aeruginosa* PA01), the latter group is of particular interest to our work.

a) Enzymes with homology to phospholipases D

The sequence homology searches have revealed that two phospho-lipolytic enzymes are putative phospholipase D enzymes. As one PLD (PldA) [26] enzyme has been previously described in *P. aeruginosa* PA01, we have consequently named two novel, putative PLD enzymes, PldB and PldC. In both enzymes, two PLD sequence motifs were identified ($HXKX_4DX_6G$), but both enzymes displayed little sequence homology to previously described PLD enzymes. The PLD superfamily is functionally heterogeneous and consists of PLD, cardiolipin synthetase, phosphatidylserine synthetase, endonucleases, helicases, and phosphodiesterase. However, the crystal structures of four members of the PLD superfamily, the endonuclease from *S. typhimurium* (Nuc) [254], the phosphodiesterase from *H. sapiens* (Tdp1) [255] and two PLD enzymes from *Y. pestis* [256] and *Streptomyces* sp. [253] show strong structural conservation of active sites consisting of two histidines, two lysines, two aspartates and two asparagines. Although active site histidines and lysines are found within common PLD motif $HXKX_4DX_6G$ motifs and are strongly conserved in the primary sequence of all PLD enzymes, active site aspartates and asparagines are located outside the PLD sequence motif and are not as strongly conserved in the primary sequence as histidines and lysines. Thus, the active site aspartates could be replaced by the glutamate or glutamine as demonstrated in the crystal structures of Nuc and Tdp1.

This example demonstrates the difference between structural conservation and primary sequence conservation. The structural conservation of biochemically equivalent residues (e.g., amino acids that can be H-bonds donors) is evolutionarily preferred and provides clear evidence about enzyme function. In contrast, primary sequence conservation is evolutionarily inferior and may allow for artefacts that are not related to proper enzymatic function. However, it appears that the PLD sequence motif, defined as $HXKX_4DX_6G$ and composed of only two (His and Lys) of four active site residues, is incomplete. Furthermore, the third active site residue (asparagine) is located close to the $HXKX_4DX_6G$ motif. Therefore, we have extended the "old" PLD motif ($HXKX_4DX_6G$) found in most literature regarding PLD enzymes to include the third active site residue ($HXKX_4DX_6GX_2N$).

The multiple sequence alignment of PldB and PldC, two PLD enzymes from *P. aeruginosa* PA01 included in the phospho-lipolytic system homologues to PLD enzymes and cardiolipin synthetase (Figure 23), shows the conservation of their active sites within two PLD sequences of homology, block I and block II. Thus, active site histidines, lysines, and asparagines of PldB were detected. The aspartate from block I corresponds to the Asp146 of PldB, but another aspartate from block II aligns with the asparagine (Asn309) of PldB. A similar conserved mutation of the catalytic site aspartate to glutamine was previously described in the structure of Tdp1 [255]. The active site histidines and lysines, and the asparagine from block II of PldC were also identified by sequence alignment. The catalytic aspartates were not conserved in the PldC sequence, and were instead replaced by glutamate (in block I) and asparagine (in block II). The mutations of the catalytic aspartates to glutamate or asparagine should not abolish catalytic activity, as the side chains of these amino acids may hydrogen bond with catalytic histidine, which is a primary function of catalytic aspartate. The most interesting finding was the prediction of catalytic asparagine from block I of PldC. The sequence alignment showed a glycine residue at the asparagine position in block I. It is very unlikely that glycine has a function similar to catalytic asparagine and therefore it may be that one of four aspartates or glutamates downstream of the $HXKX_4DX_6G$ motif could serve as a catalytic residue.

However, PldB and PldC share functional elements with enzymes from the PLD superfamily. Moreover, they have sequence homology to cardiolipin synthetase and PLD enzymes. Therefore, it is very likely that these two enzymes possess PLD or cardiolipin synthetase activity. The literature has described PLD from the pathogenic bacteria, *Y. pestis* and *C. ovis*, to contribute to the virulence of the bacteria. Furthermore, PldA from *P. aeruginosa* PA01 was found to play a role in the persistence of the bacteria in a rat model of chronic infection. Different eukaryotic PLD enzymes have ubiquitous functions, including involvement in meiosis in yeast [330], mitochondrial fusion [331], secretory vesicle fusion [332] and phagocytosis of *M. tuberculosis* [51]. These data indicate that PldB and PldC, putative PLD enzymes from *P. aeruginosa* PA01, could be potential virulence factors or have other important cellular functions.

Cellular localisation predictions indicate putative membrane association of both PLD enzymes. Neither PldB nor PldC has a signal peptide but they have a putative single transmembrane helix, therefore they are likely anchored to the inner bacterial membrane with. The prediction of catalytic domain orientation showed possible cytoplasmic or periplasmic localisation of the N-terminal catalytic domain of PldB, and the periplasmic exposure of the C-terminal catalytic domain of PldC. The membrane localisation of PLD enzymes is not uncommon because these enzymes hydrolyse membrane phospholipids, and so localisation in the vicinity of substrates allows the proteins to be accessible to the substrate. Homologues of PldB and PldC have been shown to be localised to the membrane; BpCLS is a membrane-associated protein [250], EcCLS is localised at the periplasmic site of the inner membrane [333], and mammalian PLD2 [334] is localised in the membrane. PLD of *Ricinus communis* is an example of an enzyme localised both in the membrane and cytosol, with dynamic relative distribution in these two cell compartments [335]. However, PLD from *P. aeruginosa* PA01 (PldA) is a periplasmic localised protein. Therefore, the putative membrane localisation of PldB and PldC is probable, but it should be experimentally confirmed, as examples of soluble PLD enzymes have also been described.

Although properties of PldB and PldC and PLD enzymes in general are attractive, and there is less literature on prokaryotic PLD enzymes, these enzymes will be of lowest priority in this project.

b) Enzymes with homology to esterases and lipases but without homology to PLA enzymes

The subgroup of three phospho-lipolytic enzymes composed of PlaK, EstB and EstC was identified by homology to esterases and lipases and lack of homology to phospholipase A enzymes. We screened the *P. aeruginosa* PA01 genome for esterases, lipases and phospholipases because we wanted to cover all enzymes that could possess phospholipase A or B activity. Such an approach can obviously result in hits that represent enzymes exclusively homologous to esterases but not homologous to any phospholipase A enzyme. It must be added that although enzymes may not possess homology to phospholipases, it cannot be excluded that they could be phospholipase A enzymes.

One of three enzymes from this subgroup, PlaK, will be discussed in detail later in the text; consequently, in this section, only EstB and EstC will be discussed. Both enzymes have in common a putative α/β-hydrolase fold, a GXSXG catalytic motif, a serine hydrolase catalytic triad and homology to esterases. However, their sequences are unrelated and they exhibit homology to different enzyme

families: EstB is similar to hormone sensitive lipases (HSLs), and EstC is similar to S-formylglutation hydrolases (SFGHs).

HSLs are physiologically important enzymes involved in lipid metabolism in eukaryotes [264]. They are able to hydrolyse triacylglycerol and cholesterol esters, which are the major storage sources of fatty acids. Mammalian HSLs are apparently unrelated to any other lipases. The discovery of a human HSL [223] revealed its homology to a lipase of the Antarctic bacterium, *Moraxella* TA144. Subsequently, other bacterial lipases with homology to HSLs were described and none of them showed sequence similarity to other well known lipase families. Therefore, the lipase family composed of mammalian HSLs and bacterial lipases homologous to mammalian HSLs is named the HSL family.

In the literature, HSLs are described as having undetectable phospholipase activity [265], which is quite surprising considering that many other lipases hydrolyse phospholipids. It may be that in eukaryotes, phospholipases strictly participate in phospholipid metabolism, whereas HSLs participate in energy or fatty acid catabolism. However, comprehensive experimental studies about the relationship between phospholipases and HSLs have not been done.

The multiple sequence alignment of EstB from *P. aeruginosa* PA01 and its homologues with known three-dimensional structures showed conservation of the catalytic triad and oxyanion hole residues (Figure 25). Thus, the catalytic serine of EstB (Ser150) lies within the GXSAG sequence motif. The same GXSAG motif contains an alanine residue after the serine, which is a member of the oxyanion hole. The catalytic triad glutamate (Glu243) within the E/DXL sequence motif and histidine (His273) were found downstream of the catalytic serine of EstB. Close to the N-terminus of HSLs is an HG**GG** sequence motif, which comprises two glycines (in bold) that have been structurally confirmed to be involved in the stabilisation of the tetrahedral intermediate in the enzyme-substrate complex [336]. However, in EstB, $HGGA_{83}$ was detected instead of the HGGG motif, which suggests that glycine and alanine in positions 82 and 83 in the sequence are likely the oxyanion hole residues of EstB. Therefore, alanine probably replaces glycine in the oxyanion hole of EstB. This is possible because the structure of the HSL from *A. acidocaldarius* indicates that the backbone NH groups of the oxyanion hole residues are essential for stabilising interactions with the substrate.

Together, these data demonstrate the homology of EstB to triacylglycerol and cholesterol ester-hydrolysing enzymes (HSLs), which could be involved in lipid metabolism. Likely, the HSL family is distinct from the phospholipase A family, and based on the sequence analysis, it is not likely that EstB exhibits PLA activity.

The SFGH esterase family, to which likely belongs EstC, consists of prokaryotic and eukaryotic esterases that all have in common the ability to hydrolyse S-formylglutathione to glutathione and formate [261]. These esterases catalyse the second step in the detoxification of intracellular formaldehyde that is an important feature for every organism [260]. First, toxic formaldehyde in a spontaneous reaction with glutathione results in S-hydroxymethylglutathione, which is then converted to S-formylglutathione by the activity of formaldehyde dehydrogenase [337]. Although formaldehyde may be detoxified by other enzymes in addition to SFGHs, such as formaldehyde dismutases [338] and methylformate synthetases [339], SFGHs are conserved among bacteria, yeast,

humans and plants. Therefore, Harms *et al.* suggested that the SFGH detoxification pathway is universal in nature [260].

It has been previously published that two enzymes in the formaldehyde detoxification pathway were encoded in the operon in *P. denitrificans* [260]. The same was suggested for *E. coli* [340], *O. trifall* and some other plants [337]. Such physical linkage of two functional supplementary enzymes may be an advantage to an organism, similar to that described for the pyrimidine biosynthetic pathway genes [341]. The analysis of genomic sequence around *P. aeruginosa estC* revealed a gene encoding a putative formaldehyde dehydrogenase (*adhC*, PA3629) upstream of the *estC* gene. In the database of computationally predicted prokaryotic operons [342 2009], *estC* and *adhC* from *P. aeruginosa* PA01 were organised into an operon. Although AdhC from *P. aeruginosa* PA01 was not experimentally proven as a formaldehyde dehydrogenase, it displays 86% sequence similarity to the glutathion-dependent formaldehyde dehydrogenase from *E. coli* [340], which was demonstrated *in vitro* to have glutathion-dependent activity [340]. Additionally, the *E. coli* formaldehyde dehydrogenase is closely related to plant and animal formaldehyde dehydrogenases.

The sequence alignment of EstC with homologous SFGHs, whose active sites have been identified by three-dimensional structures, showed strong conservation of catalytic triad and oxyanion hole residues (Figure 24). Thus, a nucleophilic serine was detected in the conserved GH**SM**G motif (a typical GXSXG esterase motif) together with methionine, which forms the oxyanion hole. Furthermore, catalytic triad aspartate and histidine were detected in conserved **D**XFL and D**H**SYYF motifs, respectively. The second oxyanion hole residue, leucine, which contributes to the stabilisation of the tetrahedral intermediary, is located in the conserved **L**SGLTC motif (the bolded leucine is part of the oxyanion hole).

Taken together, EstC appears to possess catalytic essential residues in the same arrangement observed in the previously described SFGHs. Moreover, the *estC* gene is perhaps organised into an operon with a putative formaldehyde dehydrogenase that could be of significance for *in vivo* functioning of the two enzymes in the formaldehyde detoxification pathway. However, the precise function of EstC should be experimentally determined.

c) Enzymes with homology to phospholipase A/B enzymes

This subgroup of phospho-lipolytic enzymes, which consists of five proteins (TesA, PlaB, PlbF, PlpD and LipF) homologous to phospholipase A enzymes, was our major interest. Therefore, three enzymes from this subgroup, TesA, PlbF and PlpD, were comprehensively studied. The sequence details of TesA and PlbF will be discussed later in a separate discussion section together with experimental data.

The novel sequence features of PlpD recognised *in silico* were later experimentally confirmed and published in collaboration with a group from Marseilles (CNRS, *Institut de Microbiologie de la Mediterranee*, IBSM) [247]. Thus, here it will be discussed only briefly. PlpD was predicted as an outer membrane protein that was later confirmed. Three domains were recognised in the PlpD sequence: a patatin-like (PLP) domain, a surface antigen variable number repeat (saVNR) and a bacterial surface antigen (BSA) domain. The PLP domain is responsible for the hydrolytic activity of PlpD, and the other

two domains are involved in protein transport through the outer membrane. The latter domains, saVNR and BSA, were found in a two-partner secretion system but not in an auto-transporter secretion system. Furthermore, sequence homology between Omp85 [244, 245], a member of a two-partner secretion system, and PlpD was detected. No sequence homology between PlpD and auto-transporters was detected. All the sequence features of PlpD are in keeping with a two-partner secretion mechanism. However, in the PlpD passenger domain (PLP) and translocation domains (saVNR and BSA) are synthesised as one polypeptide. This is a typical feature of autotransporters, whereas in two-partner secreted systems, passenger and translocation domains are synthesised as two separate proteins. Therefore, we concluded that PlpD is a unique example of a hybrid secretion system that incorporates aspects of the two-partner and autotransporter systems [247].

LipF possesses sequence homology to diverse enzymes from the GDSL-hydrolase family [127]. From the sequence alignment (Figure 18) of LipF with its homologues, we recognised four sequence motifs (blocks I, II, III, V) characteristic of GDSL-hydrolases [4]. Thus, a $GDS_{38}Y$ motif instead of a GDSL motif was identified in LipF. The catalytic triad aspartate and histidine were within the $DXXH_{288}$ motif of block V. Although, global sequence alignment did not show consensus for the oxyanion hole glycine from block II a local alignment has demonstrated glycine at position 98 in LipF aligned with oxyanion hole glycines of EstA from *P. aeruginosa* [152] and PlaA from *L. pneumophila* [123]. Oxyanion hole asparagines (Asn147) was detected in block III.

LipF displays the highest sequence homology with the extracellular *A. hydrophila* lipase, which is distinguished from other lipases by its ability to catalyse acyl transfer from phospholipids to cholesterol [49]. Interestingly, in the absence of cholesterol, the acceptor in the acyl transfer reaction, the *A. hydrophila* lipase specifically hydrolyses phospholipids at the sn2 position [343]. The acyltransferase activity of the *A. hydrophila* lipase is similar to the important mammalian plasma lecithin cholesterol acyltransferase, which facilitates reverse cholesterol transport by esterifying free cholesterol on high-density lipoprotein particles and thereby modulates atherosclerosis *in vivo* [344, 345]. Although *A. hydrophila* lipase/phospholipase A2/acyltransferase was first described more than 20 years ago [346], its physiological function still remains unknown.

Another three homologues of LipF, PLA from *L. pneumophila* [123], PLA2/LysoPLA from *V. parahaemolyticus* [196], and EstA from *P. aeruginosa* [28], were confirmed as phospholipase A enzymes. Additionally, it has been published that PLA from *L. pneumophila* does not exhibit acyltransferase activity like *A. hydrophyla* lipase/acyltransferase [123]. The physiological roles of LipF homologues have been suggested: the PLA from *L. pneumophila* has a function in the detoxification of lysophospholipids [123], the lithium tolerant lipase/esterase from *A. thaliana* participates in the defence mechanism against salt stress [217], and the *P. aeruginosa* EstA and *V. parahaemolyticus* PLA2/LysoPLA promote the virulence of these bacteria [1, 196]. These data together predict a lipase and a phospholipase A activity of LipF, with the possibility that LipF may also exhibit acyltransferase activity. The suggested cellular functions of LipF homologues hint at the possibly protective (against lysophospholipids and ions) and virulence roles of LipF.

Interestingly, the homologues of LipF are transported through the outer membrane into the extracellular milieu, with the exception of the lipase from *A. thaliana*, for which localisation data do not exist. In silico localisation of LipF showed a putative signal peptide (aa 1–20) and putative

transmembrane helix (aa 5–29), suggesting periplasmic, extracellular or membrane localisation of LipF. However, homology of LipF to extracelullar proteins and identification of a putative signal peptide suggest periplasmic or extracellular localisation rather than membrane association of LipF. The extra-cytoplasmic localisation of LipF is in keeping with the putative function of LipF in protection against extracellular effectors. Furthermore, bacterial extracellular enzymes and phospholipase A enzymes [43] have been consistently identified as potential virulence factors.

The hypothetical enzyme of *P. aeruginosa* PA01 encoded by orf. PA2927 displays sequence homology to PLA from *L. pneumophila* (PlaB); therefore, we have also named it PlaB. Screening of the protein database (UniProtKB) with PlaB sequence did not identify homology to any other previously described protein. Furthermore, in the sequence of PlaB there was no sequence motif characteristic of any family described to date in the Pfam database [184]. Thus, we compared sequences of PlaB from *L. pneumophila* and *P. aeruginosa* PA01. The sequence alignment (Figure 22), which included both PlaB enzymes and putative phospholipases, homologues to PlaB from *P. aeruginosa* PA01, showed significantly higher homology in the amino terminal part of the sequence (aa 1–270 in PlaB from *P. aeruginosa* PA01) than in the carboxy terminal part (aa 271–443 in PlaB from *P. aeruginosa* PA01).

The typical Ser-hydrolase catalytic triad was published for *L. pneumophila* PlaB [249]. However, the catalytic serine lies within the atypical THSXG pentapeptide, which differs from the typical GXSXG motif, and underscores a role for the first threonine in the pentapeptide. It appears that this threonine in interactions with substrates specifies preference of the enzyme for PLA and LysoPLA substrates but not for lipase substrates. Mutating the threonine to valine modulates PLA and LysoPLA activities at different rates (73% and 50% reduction, respectively) but does not influence lipase activity. Additionally, when threonine was mutated to glycine, both PLA, LysoPLA and lipase activities were reduced by over 95%. The catalytic triad aspartate and histidine (in bold) of PlaB are embedded in the GS**D**GVV and S**H**S conserved sequence motifs. Thus, the authors have proposed a novel class of lipolytic enzymes of which PlaB from *L. pneumophila* is the first described member [249]. In the sequence alignment (Figure 22) of *P. aeruginosa* PlaB with its homologues, strongly conserved catalytic triad residues were observed. Thus, in the *P. aeruginosa* PlaB VH**S_{85}**TG, GS**D**GTV and N**H**A motifs around the catalytic serine, aspartate and histidine (in bold) were detected. It is notable that in *P. aeruginosa* PlaB, valine is at the position of the above mentioned threonine of *L. pneumophila* PlaB. Furthermore, a remarkable difference in the sequences surrounding the catalytic triad histidine of *P. aeruginosa* PlaB and *L. pneumophila* PlaB was noticed. The S**H**S sequence was proposed as a conserved motif in the *L. pneumophila* PlaB family, but in *P. aeruginosa* PlaB, an N**H**A sequence was detected. This catalytic histidine within the N**H**A motif is related to the N/D**H**L motif found in the true lipase family [47]. The catalytic aspartate, histidine and serine involved in modulating the substrate specificity of *L. pneumophila* PlaB are also conserved in the sequence of *P. aeruginosa* PlaB.

Regarding the above differences in sequences of the catalytic serine and histidine together with the previously mentioned, unrelated carboxy terminal regions of *L. pneumophila* and *P. aeruginosa* PlaB, it would be interesting to compare the PLA and lipase activities of these two enzymes.

The catalytic activity of the amino terminal domain of *L. pneumophila* PlaB (at approximately aa 1–300) was clearly demonstrated, but there was no data on the proposed function of its carboxy

terminal domain. Thus, the function of this region of the protein is still unknown. It is possible that this sequence is a second domain of PlaB unrelated to the hydrolytic function but related to protein activation, localisation, trafficking or interactions with other proteins [122]. To address this, experimental data using full-length and C-terminal truncated PlaB proteins are necessary.

The haemolytic activity of *L. pneumophila* PlaB requires almost solely the activity of PLA. Therefore, it was postulated that hydrolysis of phosphatidylcholine, the major phospholipid in the outer membrane leaflet of erythrocytes, was responsible for the haemolytic activity of PlaB [198, 249]. It was recently published that PlaB from *L. pneumophila* is required for replication and dissemination of bacteria in a guinea pig infection model [122]. Moreover, the *plaB* negative strain of *L. pneumophila* caused less inflammation and necrosis of lung tissue [122].

Taken together, *L. pneumophila* PlaB is a PLA and LysoPLA from a novel lipolytic family that represents a virulence factor for this pathogenic lung bacterium [122]. The relatedness of PlaB from *P. aeruginosa* and *L. pneumophila*, despite the notable sequence differences, highlight *P. aeruginosa* PlaB as a prominent candidate for further studies.

5.4. Optimisation of heterologous expression of phospho-lipolytic enzymes

The heterologous expression of phospho-lipolytic enzymes should give us basic information about the efficiency of protein expression. In addition to expression yield, we were interested in the molecular state of the expressed proteins. In other words, we were interested in whether the protein was expressed as a soluble protein, a membrane protein or perhaps as insoluble molecular aggregates, referred to as inclusion bodies. Furthermore, the enzymatic activities were routinely tested with the total cell lysates to determine the possible function of the expressed enzyme. As the previous section (Discussion 5.3) indicated, three enzymes, namely PlaK, TesA and PlbF, will be described separately from the other phospho-lipolytic enzymes.

Expression of proteins was performed in *E. coli*, the most widely used host for overexpression of recombinant proteins, using two different expression systems: the common P_{T7} expression system yielding strong overexpression in *E. coli* BL21 (DE3) and the P_{lac} expression system for weaker expression. The expression vectors (Table 5) based on the commercial pET22b (+) plasmid [155] and the pBR22b plasmid [161] were used for P_{T7} controlled expression. For expression under the control of P_{lac}, expression vectors based on the pBBR1mcs-3 plasmid were used (Table 5). The *E. coli* BL21 (DE3) strain has a chromosomally encoded bacteriophage T7 RNA polymerase, which is highly selective for its own promoters and does not occur naturally in *E. coli*. The pET22b (+) multicopy plasmid that encodes P_{T7} and the natural transcription terminator from T7 DNA provides high-level transcription in the presence of a relatively small amount of T7 RNA polymerase. Using this system, greater than 50% of the desired protein from the total cell protein may be obtained in three hours or less [155]. The disadvantage of the system is the low basal level of T7 RNA polymerase in uninduced cells because total repression is not possible. The continuous translation from the plasmids carrying toxic target genes may make the plasmid unstable and could result in lack of expression.

Two different inducers, lactose and IPTG, were used for induction of P_{T7} controlled expression. Lactose and IPTG, which is a structural analogue of lactose that cannot be metabolised in the cell,

bind to the repressor of the P_{lac} operator, thereby preventing transcription by the T7 RNA polymerase. Accordingly, we induced expression by adding IPTG to the bacterial cultures in log-phase growth (see methods ddd) or by using automatic induction (autoinduction) in glucose-lactose media upon depletion of glucose. Glucose is the preferable carbon source, and once exhausted, cells use lactose as the primary carbon source. This results in synthesis of T7 RNA polymerase and the induction of protein expression. The decrease in transcription from the P_{T7} in the presence of glucose is known as catabolic repression, and in this way, glucose reduces basal expression in the beginning phase of growth, but after glucose is depleted, the induction of expression occurs. Therefore, protein overexpression was always performed in media containing glucose, LB media supplemented with 0.4% (w/v) glucose or autoinduction media containing 0.2% (w/v) glucose.

Although *E. coli* has been the workhorse for the expression of recombinant proteins, it is not a good organism for expression of membrane proteins [288], proteins with complex disulfide bridges or proteins that require posttranslational modifications [347, 348]. High levels of protein expression can trigger the bacterial proteolytic response, which degrades recombinant proteins [286]. Another effect observed as a result of high protein expression is generation of misfolded proteins, which aggregate into insoluble inclusion bodies [349]. The macromolecular crowding in the cytoplasm when protein concentrations reach up to 300 mg/ml is a highly unfavourable environment for protein folding [349]. One of the approaches to minimise aggregation is to reduce the rate of expression using a weaker promoter like P_{lac} [287]. Therefore, we used P_{lac}-controlled expression in *E. coli* DH5α for expression of proteins that produced inclusion bodies or low yields in the P_{T7} expression system. The constitutive expression of genes occurs under the control of P_{lac} without induction; however, P_{lac} can be induced in *E. coli* in presence of IPTG; thus, P_{lac}-controlled expression of proteins may be regulated with IPTG [348].

We expressed 10 phospho-lipolytic enzymes in *E. coli* and observed four general effects already described for heterologous expression of various proteins [348]. The formation of inclusion bodies was reported for LipF, PlaB and PlpD. The PlbF, EstC and PldC proteins were expressed at low levels, while no expression at all was observed for PldB and EstB. The expression of soluble and catalytically active proteins in high amounts was obtained only for TesA and PlaK. These data indicate that expression of lipolytic enzymes is difficult and probably due to the hydrophobic regions necessary for interaction with nonpolar lipids.

Enzymes not expressed

PldB and EstB were not expressed in either *E. coli* BL21(DE3) or *E. coli* DH5α and thus were not available for identification on a standard Coomassie stained SDS PAGE gel (Chapter 4.3.2). Furthermore, the total cell extracts of *E. coli* cells expressing PldB and EstB were assessed for lipase (pNPP) and esterase (pNPC) activity, but no increase in activity was observed in comparison to cells transformed with the empty vector. However, PldB was predicted to be a putative phospholipase D, and EstB a putative lipase therefore we do not expect from PldB but we do expect from EstB to be active in lipase or esterase assay. Moreover, both enzymes are predicted as membrane-bound proteins and it is generally observed that the *E. coli* strains have not been successfully useful for expression of membrane proteins [348]; therefore, it is not surprising that this two eenzymes could not be expressed in *E. coli*. We concluded that *E. coli* is not a suitable host for the expression of these two enzymes.

This putative function of PldB and EstB in energy metabolism may suggest an essential role of these enzymes, what could be a reason for encountered difficulties of the expression of PldB and EstB in our studies.

Enzymes expressed at low levels

The enzymes expressed in *E. coli* in low amounts are the three phospho-lipolytic enzymes, EstC, PldC and PlbF. Two (EstC and PldC) were identified by SDS PAGE analysis and one (PlbF) was detected in overexpression samples by enzymatic activity. PlbF will be comprehensively discussed in a separate discussion section below; thus, only EstC and PldC will be briefly discussed here.

The recombinant EstC and PldC proteins were expressed in *E. coli* BL21(DE3) as approximately 30 kDa and 40 kDa proteins, respectively. This was confirmed by comparing the cell lysates of strains expressing EstC or PldC with the strains not expressing the enzymes (Figures 38 and 39). However, it is noticeable that relatively low amounts of EstC and PldC were produced. The esterase and lipase activity assays showed no differences between strains expressing EstC and PldC and strains not expressing these enzymes. Because it is unclear whether EstC and PldC were expressed at all in their native form in *E. coli*, we cannot draw any conclusions about whether these enzymes are esterases and/or lipases. Further experiments should clarify whether EstC and PldC are expressed as inclusion bodies or as native proteins, and additional activity assays should be performed, e.g., PLD for PldC and the SFGH assay for EstC, which is predicted to belong to the SFGH family. These studies are necessary for a better understanding of EstC and PldC; however, these enzymes are not of particular interest because of their dissimilarity to phospholipases A enzymes, which are the major focus of our studies.

Enzymes expressed as inclusion bodies

It was mentioned above that a common effect of the expression of recombinant proteins is cytoplasmic formation of insoluble protein aggregates [349]. We found the formation of inclusion bodies in *E. coli* upon expression of the three phospho-lipolytic enzymes, LipF, PlpD and PlaB. Interestingly, all three enzymes are putative membrane proteins (Chapter 4.2.3). PlpD is predicted to be an outer membrane protein, which was later confirmed [247], LipF has a predicted signal sequence and a transmembrane helix in the first 25 aa, and PlaB has two putative transmembrane helices, one in the C-terminal and one in the N-terminal region. The expression of many membrane proteins in *E. coli* results in formation of inclusion bodies [350]. This is probably due to the production of higher concentrations of protein than the systems responsible for the transport of these proteins across the inner membrane (for example the Sec translocon) can process [289]. This overloading of inner membrane translocons results in a) inefficient insertion of membrane proteins into the membrane, which permits their accumulation and aggregation in the cytoplasm, b) perturbation of the membrane proteome, leading to membrane disintegration and c) energy depletion, as secretion is driven by ATP hydrolysis. Furthermore, the partially hydrophobic nature of membrane proteins may be a reason of their low solubility in aqueous environment therefore they tend to aggregate by interaction of their hydrophobic parts.

Although LipF was expressed at high levels in *E. coli* BL21(DE3) (Figure 29A), LipF was mostly recovered in form of inclusion bodies (Figure 29B). Indeed, cells expressing LipF did not yield higher esterase or lipase activities than the cells transformed with the empty vector. Because it is very likely

(from a sequence homology perspective) that LipF exhibits one of the measured activities, we were inclined to believe that LipF was expressed as an inactive enzyme rather than that LipF was not an esterase or lipase. It is known that culture conditions, such as inducer concentration and temperature, can affect the recombinant protein product [351]. Therefore, we tested whether lower concentrations of IPTG (0.1 mM instead of 0.4 mM) and lower incubation temperatures (17˚C and 30˚C instead of 37˚C) could yield soluble LipF. The results of the SDS PAGE analysis and esterase and lipase activity assays did not show presence of soluble or active LipF. We attempted to recover LipF from inclusion bodies into a bioactive form by refolding the protein. The presence of impurities (nucleic acids, proteins and phospholipids) in inclusion bodies was found to affect aggregation of folding intermediates during the refolding process [352], and so purification of inclusion bodies prior to the refolding process improved protein refolding [352]. Based on this, we purified LipF tagged with His6-tag at the C-terminus (LipFH6) from inclusion bodies, and we used this protein for refolding experiments. LipFH6 inclusion bodies solubilised with urea were purified under denaturating conditions by metal affinity chromatography (Figure 30). Despite lower purification yield probably influenced by the low affinity of LipFH6 for Ni-NTA resin used in the purification, we were able to purify approximately 2 mg of recombinant LipFH6 from one litre of the expression culture. Although proteins solubilised by urea are in a denatured state, and His6-tag should be completely exposed for binding to the Ni-NTA resin, it cannot be excluded that protein-protein interactions may negatively affect binding of His6-tag to the metal affinity column.

Refolding was performed by diluting the urea denaturated LipFH6 solution with the respective renaturation buffers (Table 10), followed by overnight incubation. The six renaturation buffers were made with four reagents described in the literature to have a positive effect on protein folding: salt, Triton X-100, sucrose and arginine. The nonionic detergent, Triton X-100, was described as a chemical chaperon that enables proper protein folding in its native form through its hydrophobic interactions [353, 354]. Arginine was described as an inhibitor of unwanted aggregation of folding intermediates, thus facilitating protein refolding [355]. This is likely due to interaction of the positively charged guanidine group of arginine with the aromatic side chain of tryptophan residues in proteins in a manner similar to π electron-cation interactions observed in acetylcholine esterase [356] and a large number of other proteins [355, 357]. Thus, arginine may inhibit hydrophobic interactions of aromatic residues in denatured or partially folded proteins [358]. Sucrose is an osmolyte that, at low concentrations, may contribute to protein folding into a native state by preventing aggregation of folding intermediates and stabilising protein structure [359, 360]. Neutral salts, like NaCl and KCl, were reported to assist in protein refolding by binding chloride anions to the folding intermediates and thus promoting the transition to the native state [361]. The combination of the above mentioned chemical chaperons with various physical properties allowed renaturation of LipFH6 into its biologically active form (Figure 31). Optimised concentrations of Triton, sucrose and arginine in the presence of a high concentration of salts (buffer 4) showed the highest lipase activity of LipFH6. Furthermore, we observed that buffers lacking arginine yielded 3-5-fold lower lipase activity compared to buffers containing arginine. Thus, it appears that arginine is the crucial factor in maintaining successful renaturation of LipF. Of note are the unusually high numbers of aromatic residues (27, approximately 9% of the total number of residues) in the sequence of LipF, which may interact with arginine to aid in the refolding of LipFH6.

The controls of a) non-refolded LipFH6 diluted with buffer lacking Triton, sucrose and arginine and b) refolding buffers alone did not display any lipase activity. Therefore, we have concluded a LipF was successfully refolded upon interactions with components of the refolding buffer.

Taken together, we conclude that recombinant lipase F (LipF) represents a novel lipase of *P. aeruginosa* PA01 and is the third lipase described in *P. aeruginosa* PA01. Previously, were reported two extracellular lipases, LipA [23] and LipC [27], in *P. aeruginosa* PA01. They share 51% sequence similarity to each other and both required, for *in vivo* activity, the same chaperon protein, LipB [23, 27]. *LipB* is encoded in the operon with the *lipA* gene and the LipB protein is anchored to the inner membrane by its N-terminal transmembrane helix [24, 362]. Interestingly, soluble LipB, without a membrane anchor, may efficiently assist refolding of *in vitro* [363]. The same organisation of lipases and the chaperon-like proteins were described in other *gram-negative* bacteria [364]. These chaperons are called lipase-specific foldases [364], and it appears that *P. aeruginosa* LipB is promiscuous for sequence related LipA and LipC. The sequence homology of LipF to LipA and LipC is not high, at 7% and 6% identity, respectively. Additionally, the dissimilarity of LipF to the other two lipases is supported by the fact that LipA and LipC are members of the true lipase family, with a GXSXG catalytic motif and an α/β-hydrolase fold [47], while LipF belongs to the GDSL-hydrolase family [127, 4], which is characterised by an $\alpha/\beta/\alpha$-fold and a catalytic serine within the GDSL tetrapeptide (Figure 18). Thus, striking differances of LipF and another two *P. aeruginosa* lipases give a hint that LipB may not assists a LipF folding. However, whether LipB of *P. aeruginosa* may fold LipF into its biologically active form still needs to be experimentally examined.

The heterologous expression and enzymatic activity of patatin-like protein D (PlpD) was recently published in collaboration with a group from Marseilles (*Institut de Microbiologie de la Mediterranee*, CNRS) [247]. Therefore, it will be only briefly discussed here. The heterologous expression of PlpD in *E. coli* BL21 (DE3) and *E. coli* DH5α yielded a full-length protein of approximately 90 kDa (Figures 32 and 34). Low-speed centrifugation showed the presence of PlpD in inclusion bodies, but the low amount of soluble PlpD was observed on SDS PAGE gel. This indicates that PlpD might be expressed in *E. coli* in its biologically active form. Therefore, the bacterial lysates of *E. coli* strains expressing PlpD were subjected to a lipase activity assay. The lipase activity was confirmed for full-length PlpD expressed under the control of P_{T7} and P_{lac}.

The size of full-length PlpD, determined by comparing the migration of PlpD with the protein standards on an SDS PAGE gel, is higher than the theoretical Mw of full-length PlpD (80.9 kDa). The bioinformatic study showed a putative C-terminal outer membrane domain (approximately 47 kDa) and a putative N-terminal catalytic, patatin-like domain (approximately 33 kDa) in PlpD (Chapter 4.2.2). Furthermore, it was demonstrated that in *P. aeruginosa* PA01 PlpD is cleaved into two peptides, each of approximately 45 kDa [247], which is in agreement with the size of full-length PlpD expressed in *E. coli*. In the same study, it was shown that the C-terminal domain of PlpD is folded in the outer membrane of *P. aeruginosa* and is responsible for transport of the N-terminal domain, which harbours lipase activity. Consequently, the N-terminal lipase domain of PlpD was detected in the extracellular space of *P. aeruginosa* PA01. Thus, PlpD was proposed as an example of a novel subgroup of autotransporte proteins, discussed previously (Chapter 5.3).

The expression of the outer membrane proteins in *E. coli* is unfavourable [289], as *P. aeruginosa* EstA expressed in *E. coli* resulted in inclusion bodies [197]. However, the recently published crystal structure of full-length *P. aeruginosa* EstA demonstrated that properly folded EstA was expressed at a low rate in *E. coli* membranes [152]. Similar to EstA, it appears that a portion of PlpD expressed in *E. coli* was properly inserted in the membrane and resulted in the formation of a catalytically active protein. The moderate expression of PlpD (under P_{lac}) yielded just 25% lower absolute activity than strong expression (under P_{T7}), although the amount of produced PlpD under P_{lac} was many fold lower than under P_{T7}. It is in keeping with general observation that a slower rate of synthesis may favour protein transport and folding [289] and production of soluble protein [286].

The catalytic domain of PlpD is released from the membrane domain in *P. aeruginosa*, but it remains covalently bound to the membrane domain after expression in *E. coli*. Thus, there is a significant difference between the recombinant and native PlpD protein. This data indicates rather weak than strong interactions of the outer membrane domain and the active site region of the PlpD catalytic domain. The strong interactions might result in the buried active site for substrate binding, thereby abolishing enzymatic activity, which was not observed for PlpD.

Although different patatin-like proteins were described to possess PLA2 activity [108, 365], our attempts to demonstrate PLA2 activity in cell lysates of *E. coli* expressing PlpD were not successful (data not shown). However, it is perhaps due to the low preference of PlpD for the PLA2 substrate used in our assay. Additional experiments are necessary to confirm our assumption that PlpD does not exhibit PLA2 activity.

In conclusion, we can state that a novel extracellular lipase of *P. aeruginosa* PA01 was discovered and that details of its expression, enzymatic activity and type of protein transport were described [247]. The association of PlpD with *P. aeruginosa* PA01 virulence, the role of the surface antigen VNR domain detected in PlpD and the processing of PlpD still remain unknown, making PlpD a good candidate for further investigation.

The heterologous expression of PlaB under different conditions has yielded sufficient amounts of recombinant PlaB for useful detection by standard SDS PAGE analysis (Figure 36). However, the amount of native PlaB in cell lysates was not sufficient to demonstrate its esterase, lipase or PLA activity. This might be due to the formation of PlaB inclusion bodies observed after slow centrifugation and SDS PAGE analysis (Figure 36). The second reason might be that PlaB localised improperly in *E. coli*; PlaB was predicted to be a membrane protein, and so improper localisation could lead to the formation of inactive enzyme. However, we demonstrated in a reaction of membrane fraction of strains expressing PlaB with a PLA2 substrate, that PlaB is likely a PLA enzyme (Figure 37). The many experiments with PlaB expressed in *E. coli* cells demonstrated only low PLA activity. As *E. coli* cells transformed with empty vector do not display background PLA activity, the measured activity of *E. coli* cells expressing PlaB may be directly related to the PLA activity of PlaB. Missing a purified PlaB it could not be confirmed PLA activity of PlaB enzyme. Taken together, the membrane localisation of PlaB, the presence of inclusion bodies in PlaB expression samples and the low PLA activity of PlaB may indicate fewer amounts of biologically functional protein expressed in *E. coli*. However, further experiments are required to precisely determine the cellular localisation and substrate specificity of this PLA enzyme described for first time in *P. aeruginosa* PA01.

It was recently published that the outer membrane PLA from *L. pneumophila* [198] participated in the virulence of this pathogenic bacterium [122]. This enzyme is homologous to the *P. aeruginosa* PlaB analysed herein, as was previously discussed (Page ss). Interestingly, transport of PlaB to the outer membrane was independent of Type I, Type II and Type IVB secretion systems [122]. Furthermore, in the protein sequence of *L. pneumophila*, PlaB was not recognised as containing either an auto-transporter motif or an outer membrane motif typical of two-partner secretion systems [122]. Moreover, the Sec or TAT signal peptide was not recognised in *L. pneumophila* PlaB [122]. Thus, the transport of *L. pneumophila* PlaB is still unclear and appears to differ from previously described lipases (LipA and LipB), phospholipase C (PlcA) and phospholipase A enzymes (PlaB and PlaC) from *L. pneumophila* [366]. The finding that *P. aeruginosa* PlaB expressed in *E. coli* is probably membrane localised, together with its homology to outer membrane PlaB from *L. pneumophila*, strength previously suggested membrane localisation of *P. aeruginosa* PlaB. *In silico* methods have also predicted membrane localisation of *P. aeruginosa* PlaB based on the two putative transmembrane helices, one in the C-terminal and one in the N-terminal region. Although it is unlikely that PlaB attaches to the membrane using both terminal sequences, it might be that one transmembrane helix anchors PlaB to the membrane. These interesting localisation features of PlaB, in addition to the PLA activity demonstrated for PlaB and its relatedness to the novel virulence factor of *L. pneumophila* [122], highlight this particular enzyme of *P. aeruginosa* PA01 as a good candidate for further studies.

5.5. First phospholipase A enzymes of *P. aeruginosa* PA01

Among the 10 enzymes selected and studied in this PhD work, three have especially attracted our attention. These three are the putative esterase, PlaK, the putative thioesterase, TesA, and the putative lipase, PlbF. The criteria in selecting the enzymes were related to our aim in characterising phospholipase A enzymes in *P. aeruginosa* PA01. These enzymes are associated with the pathogenicity and virulence of some bacteria [118, 99, 123, 87] but have not yet been described in *P. aeruginosa* PA01. Furthermore, in selecting candidates for further study, we were guided by the ability of the enzyme to be expressed as a catalitically active protein.

5.5.1. PlaK is a promiscuous enzyme with esterase, β-lactamase and phospholipase A activities

The motivation to study PlaK was that PlaK might possess both esterase and β-lactamase activities. This hypothesis was based on a) pronounced sequence homology of PlaK to group C of β-lactamases, especially in the active site regions (Results 4.2.2), and b) demonstrated esterase activity of PlaK when the enzyme was expressed in *E. coli* (Results 4.3.2).

In addition to the homology of PlaK to serine β-lactamases, PlaK has homology to the esterase (EstB) from *B. gladioli* [205]. Both *P. aeruginosa* PlaK and *B. gladioli* EstB belong to family VIII of the bacterial lipolytic enzymes [47]. This family shares the catalytic serine motif (SXXK) [47] and tyrosine, that activate catalytic serine for nucleophilic attack, with class C β-lactamases [261, 204]. However, the conserved KTG motif of class C β-lactamases was not detected in lipolytic enzymes from family VIII. The three-dimensional structure of β-lactamase from *C. freundii* shows KTG residues in the

vicinity of the active site serine and thus interacting via the H-bonds with the substrate moiety bound to the active site [206]. Therefore, their function is probably related to enzyme substrate specificity. As we expected, sequence alignment of PlaK and *B. gladioli* EstB with homologous β-lactamase displays conservation of the SXXK motif and active site tyrosine, but the KTG motif is missing in PlaK and EstB (Figure 16).

Although lipolytic family VIII does not comprise many members and a limited number are well-characterised, for some of them data about esterase and β-lactamase activity exist. *B. gladioli* EstB [367] and two esterases found in a metagenomic study [368] were negative in β-lactamase assays. To our knowledge, the only described family VIII esterase with β-lactamase activity is EstC from a leachate metagenome library [369]. However, it must be mentioned that the relative β-lactamase activity of this enzyme is 1.8% of its esterase activity. The strong preference of EstC for the ester substrate may indicate that its active site is more closely related to esterases than β-lactamases. Therefore, the authors proposed promiscuity of EstC [369], but this should be further verified. It is now thought that pronounced esterolytic activity, lack of β-lactamase activity and sequence similarity to β-lactamases are characteristic features of family VIII lipolytic enzymes.

With the aid of the X-ray structure of *B. gladioli* EstB [205], the first and only available structure of family VIII lipolytic enzyme, active site steric factors were proposed to distinguish esterases from β-lactamases. It was mentioned above that EstB from *B. gladioli* possesses esterase activity but it lacks β-lactamase activity [367]. To explain the factors preventing EstB from hydrolysing β-lactam substrates, the active sites of EstB and β-lactamase from *E. cloacae* were compared (Figure 66) [205]. Although these two enzymes display significant similarity overall, the three-dimensional structure of the shape of their active site cavities are considerably different. Figure 66 (modified from [205]) shows the proposed active sites tunnels (blue-shaded surface) that should accommodate substrate. There is a notable difference in the width (indicated by blue lines and arrows) of EstB and β-lactamase tunnels. The β-lactamase tunnel widens toward the top where the substrate sits (indicated by green arrow), whereas the EstB tunnel appears to be narrower. Furthermore, a second possible entrance to the tunnel, lying on the axis perpendicular to the figure (indicated by yellow dot), was proposed for EstB. This observation, together with the modelling studies on EstB [205], suggest that steric factors limit the selectivity of EstB to ester substrates (less bulky) instead of more bulky β-lactam substrates. However, more comprehensive structural data are necessary for identifying differences between family VIII esterases and β-lactamases.

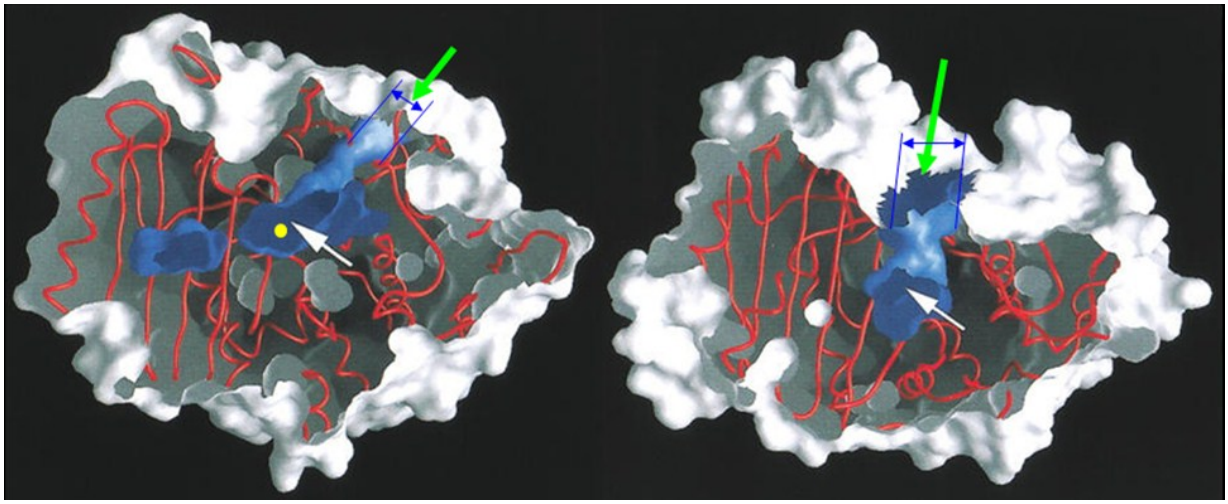


Figure 66: A section through the surface representations of EstB from *B. gladioli* (left) and β-lactamase from *E. cloacae* (right). Both enzymes are in the same orientation, and the surface is cut approximately at the height of the active site (indicated by white arrow). The active site tunnels are represented in blue. The width of the tunnel is indicated by blue lines and arrows, and the entrance of the substrate is indicated by green arrows. Modified figure from Wagner *et al.* (2002) [205].

In addition to the intriguing structure-function relationships of PlaK, a member of lipolytic family VIII [202] and β-lactamase class C, there is another interesting feature of PlaK; its putative involvement in the resistance of *P. aeruginosa* PA01. The intrinsic resistance of *P. aeruginosa* to widely used β-lactam antibiotics and the rapid development of resistance to diverse classes of antibiotics (multi-resistance) have been the subject of numerous studies in the last decade, recently reviewed in [370]. Of the different resistance mechanisms used by *P. aeruginosa* against β-lactam antibiotics, the most common is the production of β-lactamases, which inactivate and modify antibiotics [204, 291, 371]. A significant number of β-lactamases of all types are found in *P. aeruginosa* [370]. Even though it is beyond the scope of this paper, we will mention the chromosomally encoded class C β-lactamase, AmpC, responsible for the natural susceptibility of *P. aeruginosa* to β-lactam antibiotics. AmpC is constitutively expressed at low levels but may be 100–1000 fold increased by β-lactam antibiotics [370]. The regulation of *ampC* transcription is both positively and negatively controlled by AmpR. The UDP-MurNac-pentapeptide, a precursor in peptidoglycan synthesis, interacts with AmpR, which is incapable of AmpC activation in this form. When levels of the cell wall degradation product, anhydro-UDP-MurNac-pentapeptide, are increased by the action of β-lactam antibiotics, AmpR is converted to an activator of AmpC transcription, thus increasing the amount of AmpC [204]. Regulation of the bacterial response to β-lactam antibiotics through AmpC is even more complex [6]. However, chromosomally encoded *ampC* represents a dependable way of *P. aeruginosa* to develop a fast response to β-lactam antibiotics.

Our approach to biochemically characterising PlaK involved the purification of PlaK and determination of its enzymatic activities against a spectrum of hydrolytic substrates.

As previously mentioned, *E. coli* BL21(DE3) cells expressing PlaK exhibit significantly higher esterase activity (measured against pNPC) than cells transformed with empty vector (Figure 35B). SDS PAGE analysis revealed expression of a soluble protein of ca. 40 kDa upon induction of PlaK expression

(Figure 35A), which is in keeping with the molecular weight of 42.3 kDa calculated from the primary sequence of PlaK. Furthermore, we did not observe significant differences in expression, solubility and esterase activity of PlaK tagged with C-terminal His6-tag (PlaKH6) and native PlaK. PlaKH6 was purified by metal affinity chromatography using Ni-NTA resin from a soluble cell extract of *E. coli* BL21(DE3) expressing PlaKH6 (Figure 41A). However, the PlaKH6 co-eluted with a small amount of higher molecular weight proteins, as assessed by SDS-PAGE (Figure 41B). The size of these bands at ca. 90 kDa and 130 kDa suggest that they are putative dimeric and trimeric forms of PlaK. These impurities displayed an unusually high affinity for Ni-NTA resin. Fractions eluted with 100 mM and 250 mM imidazole had intensities of these impurities proportional to the amount of eluted 40 kDa PlaKH6, further supporting specific rather than nonspecific binding of these proteins to Ni-NTA resin. Additional evidence for multimerisation of PlaK came from Western blots of the purified PlaKH6 fraction with anti-His6-tag antibodies (Figure 41C). A strong signal from the monomeric PlaKH6 was seen by Western blot, but additional weak bands were detected at molecular weights corresponding to the putative dimeric and trimeric PlaKH6. Given the specificity of binding of these impurities to Ni-NTA resin, and the size of the bands detected both by SDS-PAGE and Western blots with anti-His6-tag antibodies, it is probable that these proteins are multimerised PlaKH6. Additional experiments such as separation by size exclusion chromatography may confirm the presence of multimerised PlaKH6 in the purified PlaKH6 sample.

What, then, is the nature of PlaK association in the putative dimers and trimers? It seems that multimers are partially stable under the SDS denaturing and DTT reducing conditions. One possible explanation may be the presence of five cysteine residues in PlaK molecule. DTT-resistant intermolecular disulphide bridges could form buried between two protein molecules, such as those in multimeric enzymes like human chorionic gonadotropin [372], fibronectin [373] and immunoglobulin E [374]. Further experiments are required to provide support for this theory and to reveal the structural and functional role of cysteines in PlaK.

For the purposes of our experiments, it was necessary to obtain pure enzyme in order to exclude the possibility of other interfering enzymatic activities. Recombinant PlaKH6, present as the major single band as assessed by Coomassie staining of an SDS PAGE gel (Figure 41B), was deemed to be of sufficient purity to examine its esterase, β-lactamase and phospholipase A activities. These activities were assessed using standard substrates for the detection of enzymes from the hydrolase family (see methods 3.11). All activity assays were performed with the same preparation of purified PlaKH6 at a concentration of 0.06 mg/ml. Previous experiments with cell lysates of *E. coli* BL21(DE3) expressing PlaKH6 revealed that PlaK esterase activity was inhibited after incubation with PMSF. Therefore, PMSF-inactivated PlaK was used in each enzymatic assay as a negative control and it was not active in any of mentioned assays. Positive control enzymes, *P. aeruginosa* cell lysate in β-lactamase assay and Bee venom PLA2, were used in each of the respective assays. Esterase, β-lactamase and PLA activity assays (Figure 42) showed that PlaK was active against the chromogenic substrates, p-nitrophenyl caproate [28], CENTA [171] and dithio dihexanoyl phosphatidylcholine, respectively (3.11). PlaK had an apparent preference for the esterase substrate, with specific activities for esterase, β-lactamase and PLA2 substrate of 2900 U/mg, 730 U/mg and 150 U/mg, respectively. Thus PlaK had a significant β-lactamase activity, with a specific activity approximately 25% that of its esterase activity. In contrast, the PLA activity of PlaK was only 5% that of its esterase activity.

To date, only one family VIII esterase, from metagenom [369], has been identified that has β-lactamase in addition to esterase activity. This metagenomic esterase [369] has a specific activity of 770 U/mg measured against p-nitrophenyl-octanoate, whereas its β-lactamase specific activity, measured against nitrocefin, another common chromogenic β-lactamase substrate, was only 13.5 U/mg. Thus, the esterase activity of PlaK and metagenomic esterase [369] are comparable but their β-lactamase activities cannot be compared due to use of different substrates in assays. Based on its esterase, β-lactamase and secondary PLA2 activities, PlaK is apparently a promiscuous enzyme. To our knowledge, PlaK is the first family VIII esterase [47] with both pronounced β-lactamase activity and the residual PLA activity. These findings suggest that active site and substrate binding pocket of PlaK are able to accommodate a range of substrates. The following research therefore focuses on explaining the promiscuity of PlaK on the three dimensional level. In addition, we investigate the question of whether the metagenome esterase [369] and the PlaK esterase, both possessing β-lactamase activity, represent a possible divergence in the evolution of family VIII esterases (without β-lactamase activity) and the β-lactamase family (without esterase activity) from a multifunctional ancestor to which PlaK and metagenomic esterase [369] are related [375].

As a novel chromosomally encoded β-lactamase of *P. aeruginosa* PA01, PlaK has a potential role in the intrinsic resistance of this pathogenic bacterium. The cellular localisation of PlaK may therefore give hints as to the cellular function of PlaK. Computational methods did not predict either a signal peptide (Table 14) or a transmembrane helix (Table 15) in the sequence of PlaK, suggesting that the cytoplasm might be the native cellular compartment of PlaK.

To examine its cellular location, PlaK was expressed in *P. aeruginosa* PA01 under the control of the moderate P_{lac} and the cells were separated from the extracellular enzymes. *P. aeruginosa* PA01 transformed with empty vector was used as a negative control. The extracellular and cell associated fractions were assayed for esterase activity. There was a 2.5 fold increase in cell associated esterase activity in the PlaK expression strain (Figure 44), whereas esterase activity in the extracellular fraction did not significantly differ from the control. We concluded that catalytically active PlaK is expressed in *P. aeruginosa* PA01 as a cell associated enzyme.

To determine whether or not PlaK is a soluble protein, the membranes were removed from inclusion bodies in a total cell-free lysate of *P. aeruginosa* PA01 expressing PlaKH6 The His6-tagged PlaK was chosen because it allows immunodetection of expressed PlaKH6 in each cell compartment. The empty vector strain was used as a negative control. As assessed by Western blot, PlaKH6 was found exclusively as a soluble protein without detection of even minor amounts of PlaKH6 in the membrane or extracellular fractions (Figure 44). The localisation experiments showed identical results at both middle logarithmic and early stationary growth phases. The periplasmic protein, DsbA [179], was used as a positive control for the soluble fraction. As expected, DsbA was detected in the soluble fraction, but a detectable amount of extracellular DsbA was also seen (Figure 44). One possible explanation is that there is some leakage of periplasm to the extracellular space in the PlaK expression strain. This suggests that PlaKH6 is not a periplasmic protein due to its absence in the culture supernatant. Given these results, PlaKH6 expressed in the logarithmic and stationary growth phases of *P. aeruginosa* PA01 is most likely a soluble protein of the cell cystosol. Further support for the cytoplasmic localisation of PlaK in *P. aeruginosa* PA01 came from studies in an diploma thesis, carried out under my supervision, which aimed to physiologically characterise PlaK [376]. In

mentioned work antibodies were raised against pure PlaKH6 and used for the detection of PlaK, without a His6-tag, expressed in *P. aeruginosa* PA01 under the control of P_{lac} [376]. The results demonstrated that PlaK is present in the cytoplasm but not in the periplasm (Figure 67). The periplasmic protein, DsbA, was detected only in the periplasmic fraction, demonstrating the quality of fractionation. Native PlaK was not detected in the membrane or extracellular fractions.

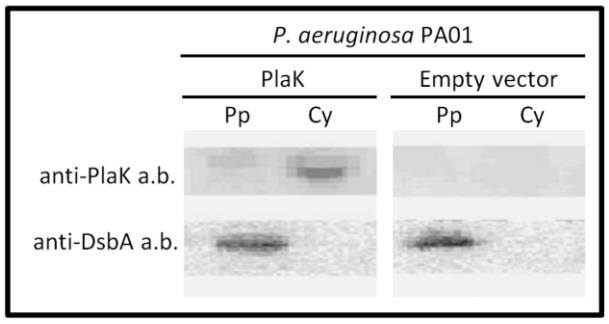


Figure 67: Western blot of localisation of native PlaK in *P. aeruginosa* PA01. The periplasm (Pp) and cytoplasm (Cy) of cells transformed with pBBR-PlaK (PlaK) and pBBR1mcs-3 (empty vector) were separated and analysed by Western blot. PlaK and DsbA were detected with anti-PlaK and anti-DsbA anti bodies, respectively. Adopted from diploma thesis of A. Mandrysch (2009) [376].

Based on cellular localisation, PlaK is more similar to EstB from *B. gladioli*, localised in the cytoplasm, than to periplasmic β-lactamases such as AmpC [377]. The periplasmic localisation of β-lactamases is related to their biological function in the detoxification of β-lactam antibiotics, which penetrate the periplasm through outer membrane porin channels and inhibit periplasmic peptidoglycan-assembling transpeptidases [291]. The biological function of *B. gladioli* EstB has not yet been proposed. Thus, we can only speculate that PlaK may play a role in the antibiotic resistance of *P. aeruginosa* or perhaps in lipid metabolism as an esterase and phospholipase A.

In the localisation study of native PlaK [376], there was no detectable expression of PlaK in the *P. aeruginosa* PA01 transformed with empty vector. Moreover, in the *P. aeruginosa* PA01 wildtype grown under standard conditions, PlaK was not detected by immunoblotting [376]. Therefore, it appears that wildtype *P. aeruginosa* PA01 does not express PlaK under the study conditions. The low wildtype expression level is characteristic of chromosomally encoded class C β-lactamases as previously mentioned [370]. In this regard, PlaK is similar to β-lactamases. *P. aeruginosa* PA01 may express PlaK or transport it in periplasm under the particular conditions or in the presence of unknown stimulants but this must be experimentally confirmed.

In conclusion, *P. aeruginosa* PA01 expresses a novel cytoplasmic esterase with β-lactamase and phospholipase A activities, named PlaK. To our knowledge, PlaK is the first esterase with promiscuous β-lactamase and phospholipase A activities. Further structural studies may explain the unique properties of this novel hydrolase. The multiple enzymatic functions hint at a putative function for PlaK in antibiotic resistance or lipid metabolism. However, to date there is little evidence regarding the natural substrates and biological function of PlaK. Future studies will be directed towards answering these questions.

5.5.2. TesA is a periplasmic esterase and lysophospholipase A1 of *P. aeruginosa* PA01

TesA is GDSL-hydrolase with the putative signal peptide and sequence homology to the bacterial PLA2. It was an attractive phospho-lipolytic candidate for study as it possesses a GDSL-hydrolase fold distinct from the typical α/β-hydrolase fold (Chapter 1.3), its extracellular localisation may be related to virulence in bacteria, and it has a putative phospholipase A2 activity. A classical approach was chosen to study this novel hydrolase of *P. aeruginosa* PA01. Recombinant TesA was purified in order to biochemically examine its enzymatic activities, to determine its physico-chemical properties (e.g., temperature optimum and stability) and to study TesA on a structural level. The sub-cellular localisation of TesA was investigated and will be used to discuss physiological aspects of the enzyme. The three-dimensional structure of TesA was solved in the closing stages of the preparation of this PhD manuscript and will be briefly reported in the Discussion section.

Crystal structure of TesA

A crystallisation screen with TesAH6 resulted in several conditions that yielded single protein crystals of sufficient quality for the determination of the three dimensional structure. TesAH6 crystals obtained using the sodium citrate buffer (50 mM, pH = 4.5) supplemented with 20% (w/v) polyethylene glycol (Mw = 3350, PEG3350) as a precipitant (Figure NH) were subjected to X-ray diffraction. The data were collected and refined at the Institute for Structural Biology[a], and will therefore not be discussed here. The structure shows that TesAH6 crystallizes as a homodimer (Figure 68A) in the monoclinic space group (Table S4). The structure of the monomer will be henceforth discussed. The analysis of TesAH6 structure revealed electron densities that could be ascribed to amino acids between positions 22 and 201. The 21 amino terminal amino acids and the carboxy terminal His6-tag of TesA were not detected in the crystal structure. No small molecules except water were detected in the structure.

The overall structure of TesA is typical of GDSL-hydrolase architecture, having a three-layer $\alpha/\beta/\alpha$-fold formed by parallel five-stranded central β-sheet surrounded on both sides by α-helices (Figure 68B). The putative catalytic triad (Ser30, His180, Asp177) of TesA is correctly oriented (Figure 68B) for deprotonation of the catalytic serine, a crucial step in catalysis. The proposed oxyanion whole glycine (Gly67) and asparagine (Asn96) are in suitable positions and distances for stabilisation of tetrahedral intermediate formed during the hydrolysis. The analysis of molecular surface of TesA reveals the location of catalytic serine on the bottom of the cavity (Figure 68B). Surface electrostatic potential of TesA is predominantly negative in the vicinity of the active site of TesA. Only a small hydrophobic surface near the active site of TesA was detected.

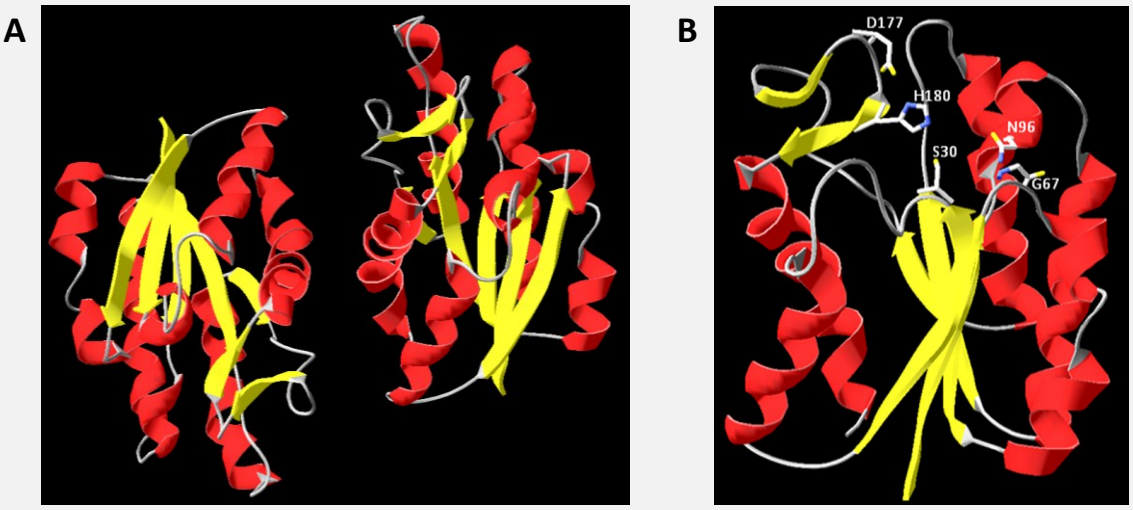


Figure 68: Crystal structure of TesA. **A)** Dimeric organization of TesA in the crystals. **B)** Overall structure of TesA with indicated active site consists of catalytic triad (Ser30, His180, Asp177) and oxyanion hole (Gly67, Asn96). Red: α-helices; yellow: β-strands. The figures are prepared with the SwissPDB Viewer program using the default settings.

[a] Renu Batra-Safferling, Joachim Granzin, Institute of structural biology 2, Research center Juelich, Germany.

Heterologous expression and purification of TesA

The expression of TesA and the C-terminally His6-tagged variant of TesA (TesAH6) in *E. coli* BL21(DE3) after induction of P_{T7} with IPTG yielded a soluble, ca. 20 kDa protein. *E. coli* BL21(DE3) cell lysates expressing TesA and TesAH6 were able to hydrolase short chain p-nitrophenyl ester (pNPC) in contrast to control cells carrying empty vector (Figures 28B). These results suggested that TesA was most likely an esterase, but purified TesA was required to confirm this. Recombinant TesAH6 was purified from soluble cell lysate by metal affinity chromatography using imidazole elution from nickel chelate column. The predominant purified protein was a band at ca. 20 kDa on SDS-PAGE. However, a second, fainter band was seen a ca. 23 kDa. To determine whether this larger band was an impurity, we excised the bands from SDS-polyacrylamide gels and analysed them by MALDI MS after the tryptic digestion. Both proteins were identified to have identical peptides characteristic of TesA, and we therefore concluded that both proteins were TesA. The putative 2 kDa signal peptide predicted in TesA (Chapter 45B) was not detected in MS analysis of either the 20 kDa or 23 kDa bands, ruling out the possibility that the larger band represented TesAH6 with uncleaved signal peptide. We have found that the use of “self made” SDS PAGE gels, such as is shown on figure 45B, sometimes results in the migration of single protein as a two distinct bands of low and high intensity. We believe that the appearance of a higher molecular weight protein band in purified TesAh6 is an artefact of the electrophoresis gel system used in the experiment and does not represent a separate protein. Therefore, the TesAH6 preparation was assumed to be of sufficient purity for biochemical, physico-chemical and structural studies.

Substrate specificity of TesA, an esterase with side lysophospholipase A1 activity

TesA shares sequence homology and conserved active site motifs with various hydrolases (thioesterases, esterases, lipases, lysophospholipases and serine protease) (Figure 17). All TesA homologues belong to the family of GDSL-hydrolases [4] that includes enzymes with diverse hydrolytic and acyltransferase activities. Whereas the global sequence similarity of GDSL-family is generally less than 20%, the family is characterised by characteristic highly conserved active site residues. Four blocks of homology, each enclosing strongly conserved catalytic triad and oxyanion hole residues [4], were recognised in TesA (Figure 17), classifying it as a member of the GDSL-hydrolase family. TesA has 44% sequence identity to two well characterised GDSL-hydrolases: thioesterase from *E. coli* (TAP) [3] and arylesterase from *V. mimicus* [212]. Both TesA homologues are esterases whereas TAP also has thioesterase, lysophospholipase and protease activities [143]. We tested whether TesA, like its homologues, has esterase, lipase, thioesterase, phospholipase and protease activities. Lipase and esterase activities were screened using p-nitrophenyl esters of unbranched fatty acids of different chain lengths ranging from two (C2) to eighteen (C18) carbon atoms [48]. TesA hydrolysed short and middle chain pNP esters (C4–C12) (Figure 47), characteristic of esterases. In contrast, TesA did not hydrolyse long chain (C16 and C18) pNP esters, the favoured substrates of lipases. TesA therefore appears to be an esterase with a substrate preference for short and middle chain fatty acid esters.

Interestingly, there was an almost five fold decrease in TesA hydrolytic activity against pNP-acetate (C2) compared to pNP-butyrate (C4). The efficiency of an enzyme in hydrolysing various substrates is determined by the specific interactions of the substrate with the enzyme surface near the active site that result in substrate binding [378]. An analysis of the TesA active site may therefore help to explain the catalytic properties of the enzyme. The crystal structure of TesA showed that the enzyme active site is mostly polar with a relatively small hydrophobic pocket (Figure 69). The hydrophobic pocket is most likely required for stabilisation of the fatty acid chain of the ester substrate and might be too small for stabilisation of long chain substrates, providing an explanation for the preference of TesA for short and middle chain p-NP esters over long chain p-NP esters. The observed decrease in catalysis of p-NP-acetate compared to p-NP-butyrate might be explained by the fact that the distance between the catalytic serine and the hydrophobic pocket of TesA may be too far for the short fatty acid chain of p-NP-acetate to interact with the hydrophobic pocket, whereas the longer butyrate chain might be better accommodated. Our studies suggest that TesA is an esterase with some atypical properties originating from the shape and charge distribution of the active site cavity. Extensive analysis of TesA structure and the co-crystalisation of TesA with the fatty acids and the inhibitors (in progress) should reveal further details on the substrate binding sites.

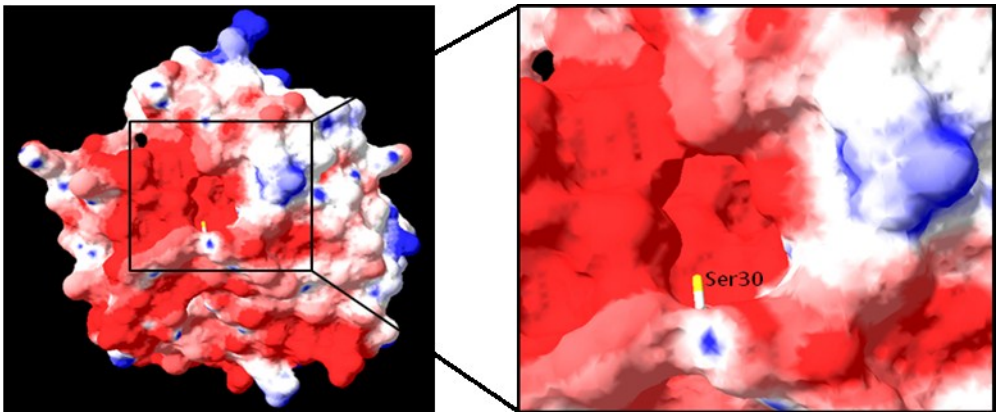


Figure 69: Representation of the molecular surface of TesA. Left: the electrostatic potential on the surface of the TesA molecule. Right: the enlarged section (right) showing the active site cavity and catalytic Ser30 with its side chain oxygen atom coloured in yellow. Red: negative potential, blue: positive potential and white: neutral potential.

A range of hydrolytic substrates characteristic for lysophospholipase A1, phospholipase A1, phospholipase A2, thioesterases and proteases was used to examine the substrate specificity of TesA. The results show that TesA has greatest activity on the LysoPLA1 (Figure 48). PLA1 and PLA2 substrates were hydrolysed by TesA 5- and 25- fold more slowly than the LysoPLA1 substrate, respectively. Thioesterase activity was not detected with TesA. The esterase activity of TesA was much stronger than its activity against LysoPLA1 and PLA substrates, with specific activities of 990 U/mg against the esterase substrate, p-NP-pentanoate, compared to 20 U/mg against the LysoPLA1 substrate, 1-palmitoyl lysophosphatidylcholine. This difference may be due to the length of fatty acid attached to the esterase and LysoPLA1 substrates, as the screen of TesA activities against a range of p-NP esters showed that TesA does not hydrolyse long chain fatty acid esters effectively. Therefore, it is not surprising that 1-palmitoyl lysophosphatidylcholine is not a good substrate of TesA and that the hydrolytic activities of TesA against 1-palmitoyl lysophosphatidylcholine and p-NP-palmitate (38 U/mg) are comparable. Additionally, the PLA1 and PLA2 substrates with two fatty acids attached to the glycerol were hydrolysed by TesA at a lower rate than the LysoPLA1 substrate. Taken together, these data support our previous conclusion that the polar active site of TesA does not favor interactions with very hydrophobic substrates. However, the small hydrophobic area in vicinity of TesA active site perhaps allow binding of short and middle chain fatty acids of esters thus resulting in their hydrolysis as it was experimentally demonstrate.

Despite the high sequence homology of TesA with the thioesterase from *E. coli* (TAP) [5], our results have shown that TesA is not a thioesterase. Additionally, unlike TesA, TAP also has proteolytic activity. That two enzymes with such high sequence identity can have such difference substrate specificities and activities was intriguing and prompted further structural investigation of the two enzymes. Several three dimensional structures of native TAP in complexes with inhibitors or substrate analogues have been solved using X-ray crystallography [379, 3] and NMR [140, 141] and these provide a vast amount of data on the substrate interaction sites of this enzyme. From these structures, Ser18 and Arg160 of TAP were proposed to be the binding site of the coenzyme A (CoA) moiety of the thioesterase substrate. Superimposition of TAP and TesA displays structural diversity in the region of the CoA binding site (Figure 70). The Ser18 and Arg160 of TAP are structurally equivalent to the Asp38 and Leu183 of TesA (Figure 70). The Ser18 probably provides an H-bond to

the CoA moiety when Arg160 likely stabilises the CoA moiety by ion-ion interactions. The negatively charged Asp38 of TesA is not an H-bond donor as is the Ser18 of TAP and Leu138 of TesA does not have the positively charged side chain of Arg160 of TAP. The surface around the CoA binding site of TAP has both hydrophobic and positively charged regions, whereas the equivalent surface of TesA is negatively charged. These structural differences of TesA and the TAP are clearly relevant to thioesterase activity and they may explain the different substrate specificities of the two enzymes. TesA and TAP thus help to confirm the importance of structural homology over the sequence homology for the determination of catalytic properties of enzymes. Sequence homology must be used with caution in the interpretation of functional homology of certain enzymes.

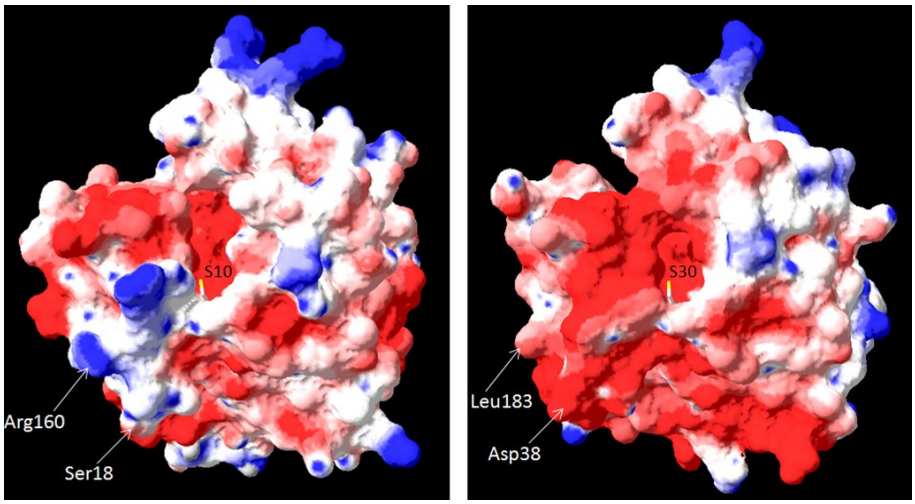


Figure 70: Electrostatic potential on the molecular surface of *E. coli* TAP [3] (left) and *P. aeruginosa* TesA (right). The catalytic serines in the active site are depicted in ball-and-stick representation with the oxygen atom of the serine side chain coloured in yellow. The difference in size and polarity of active site cavities of these two enzymes is clearly visible. The structurally equivalent residues of the TAP putative CoA binding site (Ser18 and Arg160) in TesA structure are Asp38 and Leu183. The likely inability of Asp38 and Leu183 to stabilise the negative charge of CoA molecule explains the lack of TesA thioesterase activity.

TesA contains a catalytic triad typical of GDSL-hydrolases

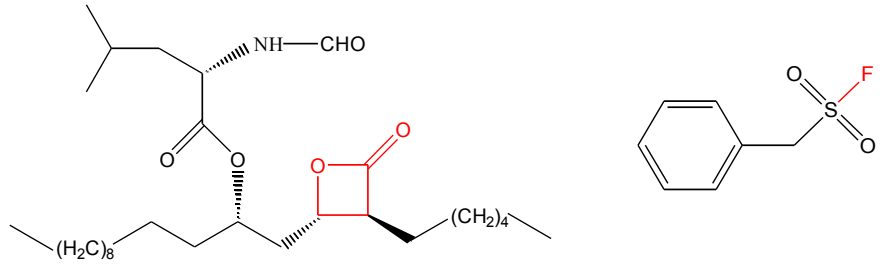
The catalytic triad (Ser18, Asp177 and His180) of TesA was predicted from the sequence alignment of TesA with GDSL-hydrolases of known three-dimensional structure and containing defined active sites (Figure 17). Site-directed mutagenesis was used to substitute an alanine for each of the residues in the catalytic triad. Alanine cannot take over the role of the nucleophilic serine or the proton acceptors, aspartate and histidine, in the hydrolytic reaction. Thus, a single mutation in the catalytic triad diminishes the crucial hydrogen bond network between Ser, His and Asp, resulting in the catalytic inactivation of the enzyme. The catalytic activities of all three catalytic triad mutants expressed in cell lysates were severely reduced compared to the wildtype TesA. Because the experiments were not performed with purified enzymes, it was not possible to precisely quantify the residual esterase activity of the catalytic triad mutants. However, the activity of catalytic triad mutants were roughly the same as an empty vector control. Furthermore, SDS PAGE analysis has confirmed expression of mutants therefore the reduction of activity is not due to lack of protein expression. These data, combined with the structural analysis data showing the three dimensional arrangement of Ser18, Asp177 and His180 favourable for the enzymatic catalysis, provide strong

support for a TesA active site comprising these three critical residues. However, the detailed role of each catalytic triad amino acid in TesA catalysis must still be elucidated using purified enzymes.

TesA is inhibited with active site serine targeting irreversible inhibitors, THL and PMSF

The inhibitory effect of two serine hydrolase inhibitors, THL [297] and PMSF [300], on the TesA was tested. The aim of inhibition was to define conditions that result in the complete inhibition of TesA activity in order to use this inhibitor for the crystallisation of an enzyme-inhibitor complex. The inhibition properties of THL and PMSF differ due to dissimilarity in the polarities of those two compounds and the difference in their reactive groups (Figure 71). THL possess two hydrophobic alkyl chains of eleven and six carbon atoms that mimic the hydrophobic lipase substrates. In contrast, PMSF is an inhibitor without any alkyl chain but with aromatic benzene ring. TesA was more strongly inhibited with THL than PMSF (Figure 49). Given that TesA displays lower activity against p-NP-acetate than p-NP-decanoate and the structural similarity of THL to the p-NP-decanoate and PMSF to p-NP-acetate, these results are not unexpected. The recognition and binding of a substrate or inhibitor are governed by the same forces. As previously discussed, the hydrophobic pocket near the active site of TesA is distant from the catalytic Ser (Figure 70) therefore esters with short chain fatty acids, like p-NP-acetate, may not bind strongly to the enzyme. In contrast, p-NP esters with longer fatty acid chains (consisting of 4 to 12 carbon atoms) bind strongly to TesA. It would therefore be expected that PMSF would bind more weakly to TesA than THL, resulting in a weaker inhibition. However, precise inhibition kinetics studies are required to provide the quantitative inactivation data that would shed light on differences in the interaction of these inhibitors and TesA. As biochemical characterisation of enzymes was not the primary objective of this PhD work, further experiments are necessary to elucidate the biochemical properties of TesA.

TesA was not completely inhibited after incubation of the enzyme with an excess of inhibitor for 1 h. However, prolonged incubation resulted in the complete inactivation of TesA with both inhibitors. Both inhibitors were therefore suitable for the preparation of covalent TesA-inhibitor complexes for use in crystallographic studies. In order to form a stable inhibitor-enzyme complex for purification, cell lysates were incubated with an excess of inhibitor overnight prior to purification to homogeneity of the TesA-inhibitor.



THL PMSF

Figure 71: The molecular structures of tetrahydrolipstatin (THL) and phenylmethylsulphonyl fluoride (PMSF) with the reactive lacton and fluoride groups represented in red.

TesA is a periplasmic enzyme of *P. aeruginosa* PA01 with unknown cellular function

In order to explore the putative role of TesA, a novel esterase and first identified LysoPLA1 of *P. aeruginosa* PA01, we determined the subcellular localisation of TesA and attempted to generate a *tesA* negative strain of *P. aeruginosa* PA01 (Δ*tesA*). Despite following a proven method for gene deletion from the *P. aeruginosa* chromosome, the *tesA* negative mutant strain was not obtained. This may indicate an essential role of TesA in the *P. aeruginosa* PA01, but to date we do not have any evidence to confirm this. Only hint is observation that *P. aeruginosa* PA01 wild type expresses TesA constitutively.

Computational analysis revealed the presence of an N-terminal signal sequence in TesA and thus predicted an extracytoplasmic localisation for this enzyme. The involvement of extracytoplasmic proteins in fundamental cellular processes like cell wall assembly, maintenance of cell membranes, nutrient uptake, energy generation, and environmental sensing, among other functions [380], underline their importance in bacteria. Extracellular proteins are generally considered to be potential virulence factors of bacterial pathogens [8]. For example, extracellular hydrolases, phospholipase C (PlcH, Chapter 1.1) and proteases (elastases LasA and LasB, alkaline protease) are virulence factors of *P. aeruginosa* PA01 [8]. In addition, type III secreted PLA from *P. aeruginosa* PA14 (ExoU), *Yersinia enterocolitica* (YplA) [100] and group A *Streptococcus* (SlaA) [43] are virulence factors of those pathogens.

The transport of proteins across the cytoplasmic membrane of *P. aeruginosa* occurs by various mechanisms [306], predominantly by recognition of N-terminal signal peptide [380]. *P. aeruginosa* has two distinct N-terminal signal peptide dependent pathways, the Sec and TAT pathways. Signal peptides are usually short sequences characterised by a tripartite structure consisting of a positively charged N-terminal sequence followed by a hydrophobic sequence and ending with a polar sequence [381]. Despite the similarity of the TAT and Sec signal peptides, a unique twin arginine motif preceding the hydrophobic region of TAT signal peptide [382] allows certain differentiation of these two types of signal peptides. The majority of tripartite signal peptides are cleaved by the signal peptidase I, which recognises the AXA tripeptide. These signal peptides are named type I signal peptides.

The first 21 amino acids of TesA are predicted to be a type I signal peptide with a typical AAA tripeptide (aa positions 19–21), probably recognised by signal peptidase I but without the twin arginine motif. Therefore, it is likely that TesA is transported via a Sec mechanism and that its signal peptide is cleaved after the translocation across the cytoplasmic membrane. Consequently, TesA may be translocated to the periplasmic or even extracellular space. To test this hypothesis experimentally, extracellular, periplasmic, membrane and cytoplasmic proteins of *P. aeruginosa* PA01 strain were separated and assayed for TesA esterase activity. *P. aeruginosa* PA01 possesses two documented esterases, outer membrane EstA [28] and intracellular esterase PA3859 [383], in addition to numerous putative esterases that perhaps contribute to the overall esterase activity of *P. aeruginosa* PA01 wild type. Therefore, we compared the esterase activity of a strain expressing TesA (moderate expression controlled by P_{lac}) with an empty vector control strain. Esterase activity was significantly increased in the periplasmic fraction when TesA was expressed. The total esterase activity of the periplasmic fraction of the TesA expressing strain was 2.5 fold higher than the activity of empty vector control strain. Esterase activity in the cytoplasmic fraction of the TesA expressing strain was

slightly elevated (ca. 25%) compared to the empty vector strain. Given the possible periplasmic localisation of TesA, the enhanced activity in the cytoplasm was likely due to an incomplete extraction of periplasmic proteins from cytoplasmic proteins.

To confirm TesA localisation, TesA activity was specifically detected by zymography. This approach allows the detection of enzymatic activity after the separation of proteins on an SDS polyacrylamide gel. After the removal of SDS, the gel was subjected to renaturation conditions under which proteins may adopt their native conformation. By overlaying those renaturated proteins with substrate, it is possible to monitor their activity. The enzymatic activities of the *P. aeruginosa* EstA, LipA and LipC [172] were previously published using the pro-fluorescent MU-esters. We first confirmed the suitability of this method for detection of TesA using purified TesA with MUB 2 h after renaturation. On the SDS polyacrylamide gel, activity of a ca. 20 kDa protein was detected in the *P. aeruginosa* PA01 subcellular fractions (Figure 51B). The strongest activity was detected in the periplasmic fraction of strain expressing TesA, but low activities were also detected in the cytoplasmic fraction of strain expressing TesA and in the periplasmic fraction of empty vector strain (Figure 51B). That the highest amount of catalytically active TesA was found in the periplasm provides further evidence for the periplasmic localisation of TesA. The detection of periplasmic TesA in the *P. aeruginosa* PA01 transformed with empty vector indicated that *P. aeruginosa* PA01 expresses TesA constitutively, as does the *P. aeruginosa* PA01 wildtype (data not shown). The tiny amount of TesA activity detected by zymography in the cytoplasm of the TesA expressing strain suggested that the cytoplasmic fraction may be contaminated with a small amount of periplasm. Further evidence for the periplasmic localisation of TesA comes from the crystal structure in which the first 21 amino acids, corresponding to the signal sequence, were not detected. This means that the putative signal peptide of TesA is cleaved off in the *E. coli* BL21(DE3) used in the crystallisation experiments, whereas cytoplasmic proteins are not processed by signal peptidases. In addition, cytoplasmic TesA is not due to an accumulation of the protein upon overexpression, as the Sec substrates are transported in their unfolded, inactive, conformation [384] and TesA present in cytoplasmic fraction is active enzyme. Therefore, all evidence supports a periplasmic localisation of TesA.

We have demonstrated that TesA is synthesised as a precursor protein and is translocated across the inner membrane, likely by the Sec secretion machinery, after which its N-terminal 21 residue signal peptide is cleaved off, probably by signal peptidase I, releasing the active enzyme into the periplasmic space of *P. aeruginosa* PA01.

Both homologues of TesA, TAP from *E. coli* [213] and arylesterase from *V. mimicus* [212] are extracytoplasmic proteins, with TAP localised to the periplasm and arylesterase to extracellular space. The natural substrate of arylesterase from *V. mimicus* [212], and consequently its physiological role, are to date unknown. Arylesterase activity was also reported for TAP [212] and TesA (against aromatic p-nitrophenyl esters, Figure 47). Periplasmic esterases are usually involved in the detoxification of xenobiotics [385] or the utilisation of nutrients [386], both of which may be potential roles of TesA *in vivo*.

Despite a long history of research on TAP protein, its physiological role is still unclear. It is thought that thioesterase and protease activities are not likely natural functions of TAP because the physiologically function of thioesterases is associated with the alteration of acyl carrier proteins

present mostly in the cytoplasm and the hydrolytic activity of TAP against these proteins is extremely low [3]. The most likely physiological function of TAP is that of a lysophospholipase as it shows specificity for sn1 position of natural lysophospholipids and its localisation allows physical contact with the phospholipids from inner and/or outer membranes [44, 387]. Even though this function was not proposed by the authors [3], in *E. coli* physiology, it is generally thought that lysophospholipases may be involved in keeping the concentration of toxic lysophospholipids below physiological levels [388]. A role in detoxification of lysophospholipids was proposed for another GDSL-hydrolase, extracellular lysophospholipase PlaA from *L. pneumophila,* protecting *L. pneumophila* against cytolytic lysophospholipids [123]. In addition to *L. pneumophila*, the cell lytic effect of lysophospholipids has been demonstrated for mycobacteria [389], Staphylococci, plant viruses [390], and mouse ascites tumour cells [391] but not for the *E. coli* cells [392]. Although the direct effect of lysophospholipids on the *P. aeruginosa* membranes is not known, the bactericidal role of eukaryotic secreted group IIa PLA2, the activity of which releases lysophospholipids, has been demonstrated in various *P. aeruginosa* clinical isolates, parallelling the hydrolysis of bacterial outer membrane lipids [393]. TesA, a periplasmic lysophospholipase of *P. aeruginosa*, may hydrolyse potentially cytotoxic lysophospholipids formed in the bacterial outer membrane by the activity of mammalian group IIa PLA2 during the infections. However, further work is necessary to elucidate the function of TesA *in vivo*.

In this section, we have presented the detection and initial biochemical and structural characterisation of TesA, a novel periplasmic esterase and lysophospholipase from *P. aeruginosa* PA01. Future studies of TesA will focus on a precise biochemical characterisation of the enzyme and the kinetics of the esterase and lysophospholipase reactions, examination of its active site structure in complex with inhibitors and fatty acids, and defining characteristic *tesA* phenotypes using a *tesA* null mutant to investigate the relationship between TesA and *P. aeruginosa* PA01 physiology.

5.5.3. Phospholipase PlbF is a novel virulence factor of *P. aeruginosa* PA01

We hypothesised that a putative phospholipase A (Figure 19) of *P. aeruginosa*, PlbF, is a potential virulence factor, and so tested PlbF for a range of lipolytic activities with the aim of defining an enzymatic function of this novel hydrolase. As PlbF has a predicted signal peptide (Table 14), we determined the sub-cellular localisation of PlbF with the aim of determining the physiological role of PlbF and its relationship to *P. aeruginosa* PA01 pathogenicity. A *plbF* negative mutant strain was also characterised.

In the diploma thesis [376] carried out under my supervision, PlbF was biochemically and physiologically characterised, and these data will be discussed together with the data obtained in course of this PhD work.

A sequence homology search revealed that PlbF had a sequence identical to both the previously described lipase, Lip3, of *P. aeruginosa* LST-03 [218] and EstA from *P. aeruginosa* 1001 [219, 220]. In contrast to our investigation of this enzyme as a potential virulence factor of this pathogenic bacteria, Lip3 and EstA were studied from a biotechnological perspective. EstA was successfully used for the production of optically pure (*R*)-β-acetylmercaptoisobutyric acid (RAM) [219], an intermediate in the synthesis of hypertension and congestive heart failure drugs (Captopril and

Alacepril) [221]. The biotechnological potential of Lip3 of *P. aeruginosa* LST-03 was examined for its resistance to organic solvents, as this strain is known to produce solvent-stable lipases [394, 395]. Lip3, however, did not show an increased resistance to organic solvents [218]. Additional information regarding the structural properties or biochemical and physiological functions of Lip3 or EstA were not provided in these biotechnological reports.

PlbF has esterase, PLB and thioesterase activities

The expression of PlbF in different expression systems (pLac and pT7 controlled) and strains (*E. coli* BL21(DE3), *E. coli* DH5α, *P. aeruginosa* PA01) yielded PlbF protein in insufficient amounts to be detected on Coomassie-stained SDS polyacrylamide gels but in sufficient quantities to be detected by esterase activity assay in crude cell lysate (Figure 40). The respective strains transformed with empty vector had much lower (40 fold) esterase activity than the PlbF expression strains. Lysates of cells expressing PlbF and of empty vector cells did not have lipase activity on the substrate, pNPP. The expression of PlbF with a carboxy-terminal His6-tag in *E. coli* and *P. aeruginosa* PA01 was confirmed by means of Western blot analysis with anti-His6-tag antibodies (Figures 58 and 62). Overexpressed PlbFH6 was purified to homogeneity from *P. aeruginosa* PA01 cell lysates using metal affinity chromatography in the presence of Triton X-100. Purified PlbF (Figure 56) had esterase but not, as was predicted by the Pseudomonas Genome Database, lipase activity and thus represents a novel *P. aeruginosa* PA01 esterase. We were interested in the substrate specificity of this novel lipolytic enzyme because it has sequence homology with phospholipases B and lysophospholipases (Table 13). However, the relatively rapid loss of enzymatic activity of purified PlbFH6 stored at -20˚C and 4˚C precluded its detailed characterisation. This instability was most likely due to the fact that Triton X-100, used in the purification to solubilise PlbF from the membranes, does not stabilise the enzyme. Therefore, the selection of a detergent that would both solubilise and stabilise PlbH6 was of the utmost importance, a common challenge in the purification of membrane proteins [396]. Due to this difficulty, PlbF expressed in *E. coli* BL21(DE3) was assayed for enzymatic activities in total cell lysates and it was found to hydrolyse phospholipase A1, phospholipase A2 and thioesterase substrates at a much higher rate than the respective empty vector strain (Figure 53).

PlbF hydrolysed diacyl-glycerophosphatydylcholine at the sn1 and sn2 positions with the roughly the same preference, what is a characteristic of phospholipases B [43], and was consequently named phospholipase B, PlbF. However, its thioesterase activity against palmitoyl-CoA was slightly higher than its PLA1 and PLA2 activities and its esterase activity was an order of magnitude higher than its thioesterase, PLA1 and PLA2 activities. Although these activities were assayed in cell lysates instead of with purified PlbF, the significantly higher thioesterase (50 fold), PLA1 (6 fold) and PLA2 (17 fold) activities of the PlbF expressing strain compared to empty vector strain led us to consider PlbF as a novel esterase with side thioesterase and PLB activities. However, the experiments using purified PlbF are needed to confirm its thioesterase and PLB activities.

Prokaryotic PLB are rare and the combination of PLB and thioesterase activities in the same enzyme have never been described to my knowledge. However, two prokaryotic thioesterases with activity against phospholipids are described to date. TAP is a thioesterase of *E. coli* with demonstrated lysophospholipase A1 activity [44]. This enzyme is able to hydrolyse 2-acyl lysophospholipids but at a lesser rate than 1-acyl lysophospholipids [44]. PLB activity in TAP was not reported. The function of lysophospholipases differs significantly from that of PLB (an enzyme with comparable PLA1 and PLA2

activities) and the sequences of TAP and PlbF are not homologous. Therefore, TAP and PlbF are unlikely to be functionally related proteins. Recently a thioesterase with phospholipase A and esterase activities was detected in *M. tuberculosis* [50]. This enzyme is involved in synthesis of mycolic acid, a key component of the *M. tuberculosis* cell wall [50]. The cell wall is integral to mycobacterial and bacterial pathogenesis in general, and so cell wall synthesis proteins are the targets of many antibacterial drugs [14]. It was speculated that *M. tuberculosis* thioesterase may be such a target [50]. However, as PlbF and *M. tuberculosis* thioesterase are not sequence homologues and the cell walls of *M. tuberculosis* and *P. aeruginosa* are markedly different, no conclusions can be drawn regarding the function of PlbF based on the activities of *M. tuberculosis* thioesterase. Phospholipase B and thioesterase activities are usually linked to the lipid hydrolysis for carbon scavenging or cell wall membrane remodelling in mammalian pathogens [213]. It is therefore likely that the function of PlbF involves host or *P. aeruginosa* lipids. The function of PlbF will be further discussed after the introduction of its subcellular localisation and physiological characterisation.

PlbF is an integral inner membrane protein

In this work, we have described the subcellular localisation of PlbF as the membrane of *P. aeruginosa* PA01. Computational methods predicted a typical N-terminal Sec signal peptide with the signal peptidase I cleavage sites (AVA_{15} or LLA_{28}) in the PlbF sequence. Based on this prediction, we first examined whether PlbF is secreted into the extracellular space of *P. aeruginosa* PA01. *P. aeruginosa* PA01 cultures expressing PlbFH6, under the control of pLac, were examined for the presence of PlbFH6 by immunodetection with anti-His6-tag antibodies in total cells and supernatants at the different growth stages (from logarithmic to stationary phase) (Figure 57). PlbFH6 was not detected using anti-His6-tag antibodies by Western blot in the supernatant but was detected in whole cells, indicating that it is a cell bound enzyme. Most of the cell-associated PlbFH6 appeared to be localised to the membrane (Figure 58). A weak PlbFH6 signal was detected in the soluble fraction containing both periplasmic and cytoplasmic proteins. The presence of a known membrane protein, XcpQ [397], in both fractions showed that membrane proteins were present in the soluble fraction. That supported the idea that the small amount of PlbFH6 detected as a soluble protein was due to incomplete separation of membrane and soluble proteins. In addition to analysis by Western blot, the same samples, along with equivalent fractions from *P. aeruginosa* PA01 expressing PlbF (without His6-tag), were assayed for esterase activity. The esterase activity of strains expressing PlbF and PlbFH6 was associated with the membranes. The membranes of empty vector strain had less than 2% of the activity of the membrane fractions of PlbF and PlbFH6 expressing strains. Additionally, the membrane activities of PlbF and PlbFH6 expressing strains were roughly the same. Whereas the soluble fractions of both the PlbF expressing and empty vector strains did not have esterase activity, the soluble fraction of the PlbFH6 expressing strain had 50% of the activity of its respective membrane fraction. This notable difference in PlbF and PlbFH6 expression is likely due to the effect of the C-terminal His6-tag on the proper insertion of PlbFH6 into the membrane. However, it is unclear whether PlbFH6 detected in the soluble fraction originated from membranes present in the soluble fraction or whether PlbFH6 was mislocalised due to its His6-tag. In the absence of a His6-tag, PlbF was undoubtedly localised to the membrane of *P. aeruginosa* PA01.

Western blot results (Figure 58A) showed approximately 10 fold more membrane bound than soluble PlbFH6, whereas the esterase activity assay (Figure 58B) was only elevated two-fold in the membrane versus soluble fraction. This inconsistency between protein concentration and enzymatic

activity of PlbFH6 indicated that PlbFH6 has higher activity at low concentrations than at high concentrations. As inclusion bodies were removed before the separation of membranes from soluble proteins (PlbFH6 was not detected in inclusion body fraction), we can exclude the presence of PlbFH6 inclusion bodies in the membrane fraction as an explanation for this observed inconsistency.

Interestingly, similar observations were made with EstA of *P. aeruginosa* 1001 [220], the sequence of which is homologues to that of PlbF. The authors observed that **a)** high and low level of EstA expression resulted in the same esterase activity measured in total cells, **b)** pure EstA at low protein concentrations had higher esterase activity compared to the EstA at high concentrations and **c)** SDS treatment of high concentrations of EstA increased esterase activity by 35%. The authors postulated that EstA aggregated at high concentrations but not at low concentrations and that the binding of the substrate to the active site of EstA was hindered in the aggregated form. They further postulated that the solubilisation effect of SDS released EstA from the aggregates, exposing the EstA active site and consequently increasing esterase activity. Although these authors did not report that EstA was localised to the membrane, it is very likely that EstA, like PlbF, is a membrane protein. If this were the case, the aggregation of EstA at high concentrations during purification could have been due to interactions of the protein's hydrophobic regions in the absence of a detergent in the purification buffer. The probability of EstA molecules interacting with each other at low protein concentrations is significantly lower than at high concentrations, therefore the esterase activity of low concentrations EstA is higher. Another possible explanation of the EstA activity-concentration relationship might lie in the membrane lipids isolated along with EstA. Membrane proteins are embedded in the lipid bilayer and are often isolated as lipid/protein complexes [396]. The crystal structure of bacteriorhodopsin was obtained from such a preparation [398]. The amount of lipids in the complex might be sufficient for the stabilisation of EstA at the low concentrations but not at the high concentrations. Thus, our PlbF results are not in conflict with the EstA data [220], and being instead complementary, implicate EstA and PlbF as the same proteins.

After concluding that PlbF was localised to the membrane, we performed a set of experiments to reveal **a)** the precise membrane localisation of PlbF in the inner or outer membranes, **b)** the peripheral or integral nature of membrane association, and **c)** its localisation in *E. coli*. Ultracentrifugation in a sucrose gradient revealed that PlbFH6 is probably associated with the inner membrane. In the fractions with different sucrose densities collected after the ultracentrifugation were XcpQ, a known outer membrane protein [180], XcpZ, a known inner membrane protein [181] and PlbFH6. The Western blot results suggested co-localisation of PlbFH6 with XcpZ rather than with XcpQ (Figure 59). However, of the 18 sucrose gradient fractions, PlbFH6 and XcpZ were detected in fractions 5-18 whereas XcpQ was detected in fractions 11-18. Quantification of Western blot signals by densitometry showed the most intense bands of XcpZ in fractions 10 and 11, whereas XcpQ was hardly detected in fractions 10 and 11 but peaked in fractions 15 and 16. PlbFH6 band intensities were three fold higher in fractions 10 and 11 (inner membrane) than in fractions 15 and 16 (outer membrane). These data support an inner membrane rather than outer membrane localisation of PlbFH6.

One explanation for the partial co-sedimentation of pLbFH6 with the outer membranes may be found in the overexpression conditions under which PlbFH6 was localised. It is possible that the higher amount of membrane associated PlbFH6 on the membrane proteome and/or membrane lipid

composition may alter membrane densities in such a way so as to raise the inner membrane density. Changes in the membrane proteome upon the deletion of *plbF* gene in the *P. aeruginosa* PA01 chromosome were confirmed by means of two dimensional gel electrophoresis [376]. Co-sedimentation of inner membrane and outer membrane proteins on a sucrose density gradient has also been reported in a localisation study of phospholipase PlaB of *L. pneumophila* [122] and the Dot/Icm type IV secretion system of *L. pneumophila* [399].

The inner membrane localisation of PlbFH6 may indicate that its putative signal peptide (15 or 28 amino acids long) (Table 14) remains uncleaved and serves as a membrane anchor as has been observed for a number of proteins [400]. PlbF is predicted to have two hydrophobic transmembrane domains, spanning amino acids 4-24 and 64-83, the former localised in signal peptide sequence. As hydrophobic transmembrane helical domains are a typical feature of integral inner membrane proteins [401], we examined whether PlbFH6 is an integral or a peripheral membrane protein. Membrane bound PlbFH6 was resistant to solubilisation reagents (sodium carbonate and urea) [402] and was solubilised by detergents (Triton X-100 and NLS), indicating that it is an integral membrane protein.

The three step biogenesis of inner membrane proteins involves recognition of a hydrophobic targeting sequence in the cytoplasm by the signal recognition particle protein that, in conjunction with a cognate membrane receptor, inserts unfolded protein into the protein conduction channel of the Sec system. From the channel, the protein is further laterally inserted in the membrane where it folds and often oligomerises into multimeric complexes [401]. Numerous inner membrane proteins, mostly transporters and channels, have been reported to be in multimeric form in vivo. These also include MscL [403], TetA [404] of *E. coli* and the protein conducting SecY complex [405]. Not all multimeric integral membrane proteins are channels, however. For example, non–channel tyrosine kinase Wzc of *E. coli* is present as a tetramer in the inner membrane [406].

We observed that PlbFH6 dimerises upon treatment of *P. aeruginosa* membranes with sodium carbonate or urea (Figure 60) and that these dimers remained membrane-bound. This suggests that denaturation induced PlbFH6 dimerisation requires the proximity of two PlbFH6 molecules in native *P. aeruginosa* membranes. The C-terminal PlbFH6 lipolytic domain, which is probably localised in the periplasm according to computational predictions, may interact with another PlbFH6 molecule upon denaturation to form a stable complex. PlbFH6 was only observed in monomeric and dimeric forms, and not as trimers, tetramers or multimers, suggesting that dimerisation occurs as the result of specific interactions between two PlbFH6 molecules rather than by a random process. Our results support in vivo interactions, perhaps between dimers, of PlbF in *P. aeruginosa* PA01 membranes.

It is generally accepted that transmembrane helices are instigators of protein-protein interactions in addition to serving as mere membrane anchors [407]. They mediate the formation of membrane protein complexes in a reversible and regulated fashion that has consequences in biological function [407]. Glycines, alanines and serines (small molecules) at the helix-helix interfaces are important for these protein interactions [408]. Two sequence motifs, GX_3G and GX_6G, where Gly may be replaced by Ala or Ser, were detected in two-thirds of transmembrane helical structures of helix-helix interacting proteins. Furthermore, these two classes of helix-helix interacting proteins differ in the geometry of the two transmembrane helices. The angle at which two transmembrane helices cross

with the GX_3G-like motif is 40˚ and, with the GX_6G-like motif, the angle is 20˚. The GX3G sequence in the transmembrane helix of glycophorin A is necessary for the formation of homodimers [409] and mutations of Gly residues in GX_6G-like motifs of class II major histocompatibility complex disrupted homodimerisation [410]. It was also demonstrated that SXXSSXXT and SXXXSSXT sequences could drive dimerisation of integral membrane proteins in bacterial inner membranes [411].

Analysis of the PlbF sequence showed two putative transmembrane helices (Table 15), the first (aa 4-24) containing the sequence **A**VAA**G**V (aa 13-18), a GXXXG-like motif (Figure 72). The interesting features of this GXXXG-like motif of PlbF are the valine residues following small residues in the GXXXG-like motif. The presence of amino acids with branched side chains (Ile, Val and Thr) has been reported in numerous transmembrane helices [407]. In thermodynamic terms, low-energy rotameric conformations of these residues have been proposed to minimise the entropy of helix-helix dimers [409]. Additionally, strong interaction of two transmembrane helices through two valine residues in **G**VXX**G**V motif was reported in glycophorin A [412]. Analogous to glycophorin A, the $\mathbf{A}_{13}$VXX**G**V sequence of PlbF could be an interaction site for helix-helix induced dimerisation. However, further experiments using site directed mutagenesis are needed to confirm this hypothesis.

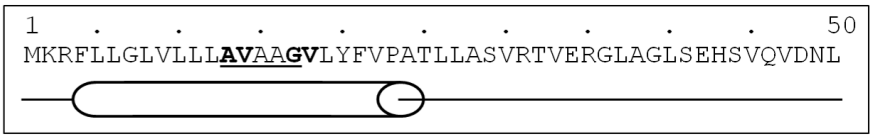


Figure 72: N-terminal sequence of PlbF (aa 1-50) with a putative transmembrane helix spanning the sequence between Phe4 and Ala24 (depicted graphically). This sequence contains the helix-helix interacting motif (GXXXG-like motif) which is underlined. Conserved **GV** (Gly can be replaced by Ala and Ser) dipeptides typically involved in helix-helix interactions are stressed in bold.

The crystal structure of an integral membrane protein, tyrosine kinase Wzc of *E. coli*, reveals its tetrameric form created by protein-protein interactions of the periplasmic domains of Wzs molecules [406]. Thus, it is not unreasonable to assume that, in vivo, interactions of the C-terminal domains of PlbF might affect enzyme activity. Such interactions in EstA of *P. aeruginosa* 1001 [220] (identical to PlbF) have been proposed to hinder the interaction of the EstA active site with its substrate. The increase of enzymatic activity at lower protein concentrations, observed for both EstA of *P. aeruginosa* 1001 and for PlbF, hint that PlbF protein-protein interactions might alter its activity. One possibility is that PlbF exists in equilibrium between monomeric and dimeric forms that is influenced by the membrane protein concentration. For example, at low PlbF concentrations, this equilibrium may be moved in the direction of the formation of a catalytically active monomer. An increase in PlbF concentration may result in the formation of inactive dimers in which catalytic domains are interacting and presumably inaccessible to substrate. Indeed, the enzymological analysis of purified PlbFH6 and PlbFH6 expressed in *P. aeruginosa* PA01 showed that the monomeric form of PlbFH6 was active (suplemary data, Figure S1). This process might have a biological role as a response of *P. aeruginosa* PA01 to stimulus by dissociation of inactive PlbFH6 dimers to active monomers rather than by the *de novo* synthesis of PlbF.

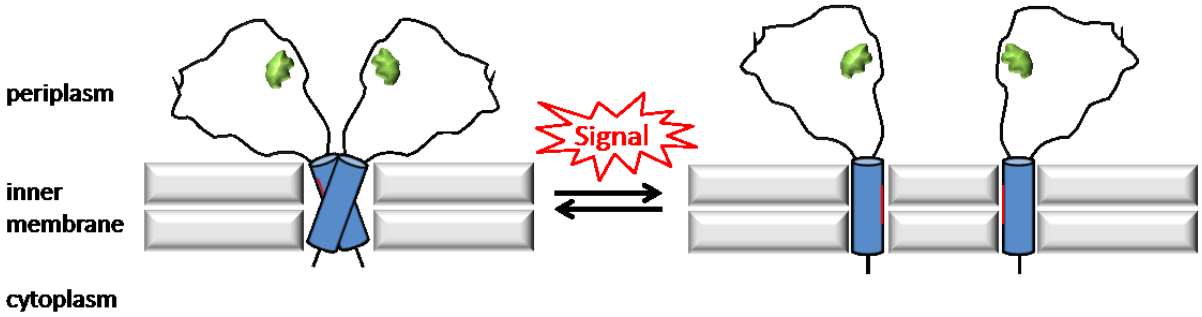


Figure 73: Model of molecular organisation of PlbF in inner membrane of *P. aeruginosa* PA01 (left) as a single transmembrane spanning integral protein. The N-terminal end of PlbF is located in the cytoplasm, the putative transmembrane helix (aa 4-24) is represented by blue cylinder, the helix-helix interacting AVXXGV motif (aa 13-18) is in red, and the C-terminal sequence of PlbF located in the periplasm comprises the catalytic domain (aa 89-302) with a putative active site (green area) consisting of Ser137, Asp286 and His258. Hypothetical mechanism explaining PlbF activation by monomerisation suggesting its possible signalling role (right).

We have demonstrated membrane localisation of catalytically active PlbFH6 in *E. coli* DH5α (Figure 62). However, the activity of PlbFH6 expressed in *E. coli* was much lower than the activity of PlbFH6 expressed in *P. aeruginosa*. These data indicate that PlbF6 is inserted into the membrane in a similar manner in these two species, probably by the evolutionarily conserved signal recognition particle and Sec systems, used for the membrane insertion of number of integral inner membrane proteins [413]. The folding of inner membrane proteins is assisted by protein chaperones [414] and lipids [415], which may affect the activities of integral membrane proteins [416]. The different lipid composition of *E. coli* and *P. aeruginosa* [417, 418], or differences in the specificity of their foldases (chaperones) for the folding of PlbF may therefore account for the observed difference in PlbF activity between these species.

PlbF is a virulence factor of *P. aeruginosa* PA01

Integral membrane proteins comprise 25-30% of all bacterial proteins [407] having pivotal roles in a plethora of cellular processes such as energy transduction, sensing and transduction of environment stimuli, uptake and efflux of molecules, adhesion and cell organisation [413, 396]. Therefore, it is not surprising that most antibiotics [419] and around 50% of drugs for a variety of diseases target membrane proteins [396]. Despite increasing research efforts, our understanding of *P. aeruginosa* virulence is still limited. The role of phospholipases A in bacterial virulence has not been fully elucidated due to the limited number of known phospholipases A.

In this work, we have demonstrated the contribution of *plbF* to the virulence of *P. aeruginosa* PA01 in a *D. melanogaster* virulence model (Figure 63). The *P. aeruginosa* PA01 strain lacking the *plbF* gene killed four fold fewer flies than the parental strain. These data point to PlbF as a novel *P. aeruginosa* PA01 virulence factor that has phospholipase B activity. A number of bacterial phospholipases A are injected into the host via the type III secretion system to the host where they develop virulence [115, 99, 101]. Type III secreted PLA2 are involved in the lysis of host cell membranes and in the modulation of the host inflammatory response. However, it is not likely that PlbF, an inner membrane protein may directly modulate host lipid signalling as the Type III effectors do.

Recently, outer membrane phospholipase A, PlaB, of *L. pneumophila* was reported to be a virulence factor [122]. The outer membrane PLA (OMPLA) of several pathogenic bacteria were also reported to be virulence factors [43]. The contribution of OMPLA to virulence is related to the processes that enhance bacterial growth and colonisation [42]. PlaB of *L. pneumophila* is thought to be similar because 20 fold less *plaB* negative mutant strain than wildtype was recovered from infected lungs [122]. The processes enhancing bacterial growth and colonisation involve alteration of the host environment by hydrolysis of membranes or lung surfactant, or by changing the composition of bacterial membranes [43]. It was sugested that *P. aeruginosa* metabolises phospholipids of lung surfactant, composed of 90% lipids and 10% proteins, which may affect bacterial growth and, indirectly, its virulence [39]. In the same study PlbF activity was induced 3-fold by phosphatidylcholine *in vitro* [39]. Besides PlbF, extracellular lipases (LipA and LipC), phospholipase C (PlcH), and fatty acid, glycerol and choline degradation enzymes were also induced by phosphatidylcholine. However, whereas many of these enzymes were induced by phosphatidylcholine *in vivo* in *P. aeruginosa* strains isolated from cystic fibrosis patients, PlbF was not induced. Although lipid metabolism might be important for *P. aeruginosa* infection, there is currently no evidence whereas PlbF is involved in these proceses.

PlbF shares sequence homology with a lipase of the psychotrophic bacteria, *Moraxella* TA144 [420] and *Psychrobacter immobilis* [202], and with human hormone sensitive lipase (HSL) [223] (Table 13). A role in the mobilisation of lipids at low temperatures for energy production was proposed for these lipases, as it was observed that **a)** PlbF homologues are active at low temperatures [223, 420], **b)** *Moraxella* TA144 lipase was not found in *Moraxella* sp. that grow at 37˚C and **c)** human HSL is not homologous to any other mammalian lipase but it is homologues to lipases from psychotrophic bacteria. PlbF retained more than 50% of its activity at 15˚C, compared to activity at its optimum growth temperature, 30˚C. In comparison, human HSL retained 70% [223], *Moraxella* TA144 lipase, 40% [420] and lipoprotein lipase and carboxyl-ester lipase, which are not assumed to be as active at low-temperatures, retained 10% and 25% of their activity, respectively [223]. One possible physiological function of PlbF might therefore be related to lipid metabolism at low temperatures or in the regulation of membrane fluidity important for bacterial growth at low temperatures. Interestingly, the contribution of PlbF to the *P. aeruginosa* virulence in the *D. melanogaster* model was assayed at 21˚C [183].

The suggested role of PlbF in lipid metabolism is not completely in keeping with the inner membrane localisation of PlbF. In bacteria, fatty acids are transported through the outer membrane, activated by inner membrane acyl-CoA synthase and catabolised via β-oxidation in cytoplasm [421]. A plausible role of esterase/phospholipase B in lipid metabolism would therefore be the hydrolysis of exogenous lipids to the fatty acids. However, as an inner membrane protein, PlbF is not in direct contact with the external environment. It is more likely that PlbF acts on *P. aeruginosa* membranes, possibly to regulate membrane fluidity at low temperatures.

There is evidence that PlbF influences biofilm formation of *P. aeruginosa* and perturbs the *P. aeruginosa* PA01 membrane proteome [376]. The *plbF* negative mutant of *P. aeruginosa* PA01 is a biofilm overproducer, and 2D gel electrophoresis comparison of the *plbF* negative mutant with the parental strain showed changes in the expression profile (5 fold or more) for at least 40 membrane proteins [376] (identification of these proteins is in progress). These data were obtained at 37˚C and

so are obviously unrelated to the proposed regulation of *P. aeruginosa* membrane fluidity at low temperatures by PlbF.

PlbF also has homology with two mouse lipolytic enzymes, monoacyl glycerol lipase (ABHD6) [230] and lysophospholipase/phospholipase B (Abh4) [231], which are involved in lipid signalling. ABHD6 is involved in the inactivation of the endocannabinoid, 2-arachidonoylglycerol (2-AG) [422]. 2-AG is biosynthesised from membrane phospholipid precursors and stimulates cannabinoid receptors (CB) in the vicinity of its own synthesis, after which it is rapidly hydrolysed by ABDH6 [230]. Endocannabinoid signalling controls numerous physiological processes from pain sensation to the maintenance of food intake (appetite) [423]. Abh4 is also involved in endocannabinoid signalling via the hydrolysis of N-acyl phosphatidylethanolamines, precursors of N-acyl ethanolamines, which are lipid mediators involved in appetite, memory and inflammation [231]. Hence, interestingly to mentioned is that *P. aeruginosa* infected cystic fibrosis patients often suffer from the disorder in food intake, an loost of appetite [424, 425]. Although these symptoms can not be attributed exclusively to *P. aeruginosa* it can not be excluded that bacteria interfere with endocannabinoid signalling in host cells. However this is only hypothesis.

The sequence homology of PlbF with ABHD6 (50% sequence similarity) is reflected in their functional homology and similar cellular localisation. Both enzymes hydrolyse phospholipids at the sn-2 position, both enzymes are inhibited completely by PMSF and partially by THL, both enzymes have high enzymatic activities typical of signalling proteins, and both enzymes are integral membrane proteins [230]. Abh4 is not as comprehensively characterised as ABHD6 but both PlbF and Abh4 have PLB activity [231].

May PlbF interfere with host cell lipid signalling? The inner membrane localisation of PlbF makes this putative function unlikely as it is not in direct contact with the host. But some of these lipid messengers are membrane diffusible molecules. Therefore PlbF might release putative diffusible lipid mesanger from *P. aeruginosa* membranes that could influence host cells or other bacteria in the population. However, this putative PlbF function should be revised and experimentally tested.

Multiple lines of observations support a role of PlbF and lipolytic enzymes in the regulation of cellular processes via lipid signalling: **a)** PlbF is active against analogues of membrane phospholipids, **b)** PlbF has sequence and functional homology with phospholipases involved in lipid signalling, **c)** PlbF is located at the inner membrane and its activity is perhaps modulated by dimerisation, a feature of integral membrane proteins involved in signalling processes [408], **d)** changes in the membrane proteome of *plbF* negative mutant strain have been observed [376], **e)** a *plbF* negative mutant strain overproduces biofilm [376], and **f)** *plbF* is induced by the addition of extracellular phosphatidylcholine to *P. aeruginosa* PA01 culture [39]. Lipid signalling and the roles of lipid mediators are comprehensively understood in eukaryotes, in which they regulate a plethora of important cellular processes [426]. Although prokaryotic lipid signalling is poorly understood, recent studies have shed light on the effect of lipid molecules on *P. aeruginosa* and in bacteria in general. For example, non-saturated fatty acids induce chemotaxis of *P. aeruginosa* [37] and dilauryl-, dioleoyl phosphatidylethanolamine induce chemotaxis of *M. xanthus* [427, 428], monopalmitoylphosphatidic acid affects virulence gene expression in *P. aeruginosa* [429], and cis-2-decanoic acid induces biofilm dispersion in a number of bacteria and in yeast [430].

Thus, inner membrane phospholipase might produce or degrade membrane bound lipid messengers that could **a)** stay incorporated in a membrane and affect the activity of an inner membrane protein, [345] consequently triggering a signalling cascade or **b)** diffuse out of membranes and affect a two-component system as diffusible sigma factor such as that mediating the pathogenicity of *X. campestris* [431] and *B. cenopcepacia* [432].

To my knowledge, there are no reports of inner membrane phospholipases B involved in bacterial pathogenesis. PlbF may therefore be involved in a novel mechanism of regulation of *P. aeruginosa* virulence, although this mechanism needs to be proofed. The data presented in this thesis on a novel virulence factor, an esterase/phospholipase B, PlbF of *P. aeruginosa* point to PlbF as a promising candidate for further studies.

6. Summary

Phospholipases A (PLA) represent a group of bacterial virulence factors with the ability to interact with host membranes and lipid signalling. Although some bacterial PLA are proven to be virulence factors, the number of known PLAs is still limited. Furthermore the details about host-pathogen interaction mechanisms remain to be elucidated. Since none PLA has been described in *P. aeruginosa* PA01 so far we wanted to examine the phospholipolytic (PLA) potential of this pathogen bacterium. For that reason we launched a comprehensive study to identify genes which might encode proteins displaying PLA activity.

We have successfully demonstrated periplasmic and membrane associated PLA activity in *P. aeruginosa* PA01. *In silico* analyses of the *P. aeruginosa* PA01 genome revealed ten promising genes, named **phospho-lipolytic** genes, which were experimentally analysed in more details.

A systematic approach encompassing sequence analysis, amplification, cloning, expression and purification of phospho-lipolytic enzymes disclosed interesting enzymatic activities for six previously uncharacterised enzymes.

Thus, two novel lipases were detected; LipF, a probably membrane bound lipase and PlpD, a patatin-like lipase, described as the fist example of a novel subgroup of the autotransporter protein family [247]. **TesA** was characterised as the first lysophospholipase A of *P. aeruginosa* PA01 in this work. Additional three enzymes **PlaK**, PlaB and **PlbF** were demonstrated to possess PLA activity. The most promising candidates among the identified phospho-lipolytic enzymes were chosen to be studied in more details, namely: PlaK, TesA and PlbF.

PlaK was predicted to be a putative esterase with a conserved β-lactamase active site motif. Thus, it belongs to family VIII of lipolytic enzymes. Despite the conservation of β-lactamase active site motif, esterases belonging to family VIII commonly exert solely esterase activity. Few of these esterases (family VIII) are described to date and only one them exhibits faint β-lactamase activity. Hence, purified PlaK was inspected for esterase, β-lactamase and PLA2 activities. Notable esterase (176 U/μl) activity of PlaK was demonstrated but also considerable β-lactamase (44 U/μl) and low PLA2 (9 U/μl) activity. Therefore, we concluded that PlaK is a unique multifunctional enzyme among bacterial hydrolases because to date an enzyme has not been described that possesses esterase, PLA2 and β-lactamase activities concomitantly. The physiological relevance of such substrate promiscuity of PlaK is still unknown. However, the combination of those enzyme activities is tempting to attribute the function of PlaK to the intrinsic resistance of *P. aeruginosa* towards β-lactam antibiotics.

TesA is a putative thioesterase of *P. aeruginosa* PA01 which function was predicted because of its high sequence homology to an *E. coli* thioesterase. Both belong to the GDSL-hydrolase family characterised by a protein fold distinct from common α/β-hydrolase fold. *E. coli* thioesterase is structurally well characterised and a multifunctional enzyme with additional esterase, lysophospholipase and protease activities. Therefore, we have examined respective enzymatic functions and structural features of TesA from *P. aeruginosa* PA01.

TesA was purified and shown to have pronounced esterase and lysophospholipase A1 activities. Despite the predicted function of TesA we could not detect any thioesterase activity, neither short nor long chain acyl-CoA esters were accepted as substrates. Consequently, TesA of *P. aeruginosa* is

not a thioesterase and in addition its protease activity could not be detected. Despite the pronounced sequence homology of *E. coli* thioesterase and *P. aeruginosa* TesA we demonstrated a striking contrast in the enzymatic functions.

In order to explain these differences, TesA was crystallised and its crystal structure was solved in the course of this work. The $\alpha/\beta/\alpha$-fold, catalytic triad and the oxyanion hole of TesA are similar to TAP and to other GDSL-hydrolases. However, comparison of molecular surface of TesA and TAP revealed remarkable differences in size and polarity of the active site cleft and particularly acyl-CoA binding site. Thereby, we have suggested a possible structural explanation of the missing thioesterase activity of TesA. The physiological function of the periplasmic localised TesA in *P. aeruginosa* PA01 remains to be elucidated but it may be involved in the detoxification of xenobiotics or lysophospholipids.

PlbF displays sequence homology to prokaryotic and eukaryotic phospholipases A, lipases and esterases involved in lipid metabolism and lipid signalling. Indeed, we were able to demonstrate esterase, phospholipase B and thioesterase activities of PlbF. Subcellular localisation experiments revealed its association with the membrane fraction of *P. aeruginosa*. Further localisation studies suggested that PlbF is anchored into the inner membrane as an integral protein. These data are in agreement with *in silico* analyses which have predicted two hydrophobic transmembrane helices in the N-terminal sequence of PlbF. Additionally, we recognised a signature of integral membrane proteins in the first helix of PlbF. This motif is a helix-helix interaction site of inner membrane proteins suggesting a dimerisation of PlbF. These findings were supported by observed dimerization of PlbF in *P. aeruginosa* PA01 membranes upon treatment with denaturants. PlbF is active as monomer but the detailed biological function of putative dimerisation cannot be completely explained at the moment. However, PlbF may be involved in signalling processes through monomerization/dimerization process and the resulting conformational changes upon the external factors.

To investigate the correlation of PlbF and *P. aeruginosa* PA01 virulence, a *plbF* negative strain of *P. aeruginosa* was constructed and compared to wild type *P. aeruginosa* PA01 in *D. melanogaster* virulence model. Significant attenuation of the *plbF* negative mutant strain in killing of flies was observed. This result confirmed that PlbF is a novel virulence factor of *P. aeruginosa* PA01. Furthermore it is the first phospholipase A2 described to contribute to the virulence of *P. aeruginosa* PA01.

Herein present comprehensive set of data is the first report about phospholipases A of *P. aeruginosa* PA01 which may represent a novel group of virulence factors. However, this knowledge is a starting point for continuative research of role of phospholipases A in virulence of *P. aeruginosa* PA01.

Summary in German

Phospholipasen A (PLA) repräsentieren eine Gruppe bakterieller Virulenzfaktoren, die mit den Membranen der Wirtsorganismen interagieren und eine Rolle beim *Lipid Signalling* spielen. Obwohl einige bakterielle PLA bereit als Virulenzfaktoren identifiziert wurden, ist die Anzahl bekannter PLA noch limitiert. Darüber hinaus müssen die Mechanismen, die bei der Interaktion zwischen dem pathogenen Organismus und dem Wirt beteiligt sind, noch genauer untersucht werden. Im Rahmen der vorliegenden Arbeit sollte das pathogene Bakterium *Pseudomonas aeruginosa* auf Enzyme mit PLA-Aktivität hin untersucht werden, da bisher noch keine PLA in *P. aeruginosa* PAO1 beschrieben wurden.

Hierzu wurde zunächst eine umfassende bioinformatische Studie durchgeführt, in der Gene identifiziert wurden, die eine PLA-Aktivität ausüben könnten. Parallel wurde experimentell gezeigt, dass in *P. aeruginosa* periplasmatische und Membran-assoziierte Proteine existieren, die eine PLA-Aktivität aufweisen. Detailliertere *in silico* Analysen des Genoms von *P. aeruginosa* PAO1 identifizierten 10 Gene, die als **phopho-lipolytische** Gene bezeichnet und experimentell genauer untersucht wurden. Im Folgenden wurden diese Gene mittels Amplifizierung, Klonierung, Expression und Reinigung untersucht. Bei den resultierenden phospholiplytischen Enzymen konnten hier erstmals sechs bisher uncharakterisierte Enzyme mit interessanten enzymatischen Aktivitäten beschrieben werden.

Es wurden zwei neue Lipasen nachgewiesen; Zum einen LipF, eine vermutlich Membran-gebundene Lipase und zum anderen PlpD, eine Patatin-ähnliche Lipase, die als erstes Beispiel für eine neue Untergruppe der Proteinfamilie der Autotransporter [132] beschrieben wurde. Darüber hinaus wurde mit TesA die erste Lysophospholipase A von *P. aeruginosa* charakterisiert. Weiterhin wurde gezeigt, dass die drei Enzyme PlaK, PlaB und PlbF PLA-Aktivität aufweisen. Die vielversprechendsten Kandidaten unter den identifizierten phospho-lipolytischen Enzymen wurden näher untersucht, als da wären: PlaK, TesA und PlbF.

PlaK wurde als putative Esterase mit einem konservierten ß-Lactamase-Sequenzmotiv im aktiven Zentrum hervorgesagt und gehört zur Familie VIII der lipolytischen Enzyme. Trotz des konservierten ß-Lactamase-Sequenzmotivs im aktiven Zentrum besitzen diese Proteine nahezu ausschließlich Esterase-Aktivität. Bis zu diesem Zeitpunkt wurden überhaupt nur wenige Esterasen der Familie VIII beschrieben und nur eine von ihnen wies geringfügige ß-Lactamase-Aktivität auf. Infolgedessen wurde PlaK auf die entsprechenden Enzymaktivitäten hin untersucht. Tatsächlich konnte demonstriert werden, dass PlaK eine beachtenswerte Esterase- (176 U/µl), aber auch eine deutliche ß-Lactamase- (44 U/µl) und eine nur geringe PLA2-Aktivität (9 U/µl) aufweist. Demzufolge ist PlaK das erste multifunktionelle Enzym unter den bakteriellen Hydrolasen, das sowohl PLA2- als auch ß-Lactamase-Aktivität besitzt. Die physiologische Bedeutung dieser Multifunktionalität von PlaK ist noch unbekannt. Dennoch lässt die Kombination solcher Enzymaktivitäten darauf schließen, dass PlaK bei der intrinsischen Resistenz von *P. aeruginosa* gegenüber β-Lactam-Antibiotika eine Rolle spielt könnte.

TesA aus *P. aeruginosa* wurde aufgrund hoher Sequenzhomologien zu einer Thioesterase aus *E. coli* (TAP) als putative Thioesterase vorhergesagt. Beide Enzyme gehören zur Familie der GDSL-Hydrolasen, die sich hinsichtlich ihrer Proteinfaltung deutlich von den üblichen α/β-Hydrolasen unterscheiden. Die Thioesterase aus *E. coli* ist strukturell gut untersucht und weist zusätzlich

Esterase- Lysophospholipase und Protease-Aktivitäten auf. Daher wurden die entsprechenden enzymatischen Funktionen und strukturellen Merkmale von TesA aus *P. aeruginosa* untersucht. TesA wurde gereinigt und ausgeprägte Esterase- und Lysophospholipase A1-Aktivität konnte detektiert werden, jedoch keinerlei Protease- oder Thioesterase-Aktivität. TesA akzeptierte weder Kurz- noch Langkettige Acyl-CoA-Ester als Substrate und ist folglich keine Thioesterase. Trotz der vorhergesagten Sequenzhomologien zwischen der Thioesterase aus *E. coli* und TesA aus *P. aeruginosa*, wurden beachtliche Unterschiede in den enzymatischen Funktionen aufgedeckt.

Um diese Unterschiede besser verstehen zu können, wurde im Rahmen dieser Arbeit TesA kristallisiert und anschließend die Kristallstruktur des Enzyms aufgelöst. TesA ähnelt bezüglich der $\alpha/\beta/\alpha$-Faltung, der katalytische Triade und der Oxyanion-Höhle dem Enzym TAP und anderen GDSL-Hydrolasen. Vergleiche der molekularen Oberfläche von TesA und TAP deckten jedoch erhebliche Unterschiede hinsichtlich der Größe und Polarität der Spalte des aktiven Zentrums insbesondere der möglichen Acyl-CoA-Bindestelle auf. Diese Unterschiede bieten eine strukturelle Erklärung für die fehlende Thioesterase-Aktivität von TesA. Die physiologische Funktion des im periplasmatischen TesA von *P. aeruginosa* muß noch aufgeklärt werden. Man kann jedoch spekulieren, dass TesA an der Entgiftung von Xenobiotika oder Lysophospholipiden in *P. aeruginosa* beteiligt ist.

PlbF weist Sequenzhomologien zu prokaryotischen und eukaryotischen Phospholipasen A, Lipasen und Esterasen auf, die am Lipid-Metabolismus aber auch dem *Lipid Signaling* beteiligt sind. In dieser Arbeit konnte jedoch erstmalig gezeigt werden, dass PlbF Esterase-, Phospholipase B- und Thioesterase-Aktivität besitzt. Untersuchungen zur subzellulären Lokalisierung von PlbF ergaben, dass PlbF als integrales Membranprotein in der inneren Membran verankert vorliegt. Diese experimentellen Befunde stimmen mit *in silico*-Analysen überein, die PlbF als Protein mit zwei hydrophoben, transmembranen Helices in der N-terminalen Sequenz von PlbF hervorgesagt haben. Zusätzlich wurde ein Motiv einer Helix-Helix-Interaktionsstelle innerer Membranproteine, die auf eine Dimerisierung von PlbF hinweist gefunden. Die Dimerisierung von PlbF wurde experimentell bestätigt und erfolgte bei Behandlung mit Denaturierungsmitteln. PlbF ist als Monomer aktiv und die biologische Funktion einer möglichen Dimerisierung kann zur Zeit nicht vollständig erklärt werden. Man kann jedoch vermuten, dass PlbF durch Monomerisierung/Dimerisierung und die dadurch resultierenden Konformationsänderungen, die durch externe Faktoren verursacht werden, an Signalübertragungen beteiligt sein kann.

Ein PlbF-negativer *P. aeruginosa* Stamm wurde konstruiert und im Vergleich zu *P. aeruginosa* PAO1 (Wildtyp) im Virulenz-Modell *D. melanogaster* untersucht. Bei der PlbF-negativen Mutante von *P. aeruginosa* wurde eine verminderte Virulenz gegenüber *D. melanogaster* beobachtet. Dieses Ergebnis zeigt dass es sich bei PlbF um einen Virulenzfaktor handelt und damit ist es die erste beschriebene Phospholipase A2, die zur Virulenz von *P. aeruginosa* beiträgt.

Zusammenfassend lässt sich festhalten, dass die umfassenden Ergebnisse, die in dieser Arbeit gezeigt wurden, darstellen dass die erstmalig beschriebenen Phospholipasen A in *P. aeruginosa* neue Virulenzfaktoren von *P. aeruginosa* repräsentieren könnten. Dieses Wissen soll als Anfangspunkt für weiterführende Studien über die Rolle von Phospholipasen A bei der Virulenz von *P. aeruginosa* dienen.

7. Supplementary data

Table S1: Putative lipolytic enzymes of *P. aeruginosa* PAO1. In bold are stressed enzymes being the subject of study in this PhD work. The data in table are adopted from Pseudomonas Genome Database.

PA code	Gene name	Product name
PA1047		**putative esterase**
PA2098		**putative esterase/deacetylase**
PA2155		**putative phospholipase**
PA2856	***tesA***	**putative acyl-CoA thioesterase I**
PA2949		**putative lipase**
PA3628		**putative esterase**
PA4339		**putative phospholipase**
PA1615		putative lipase
PA1621		putative hydrolase
PA1622		putative hydrolase
PA2411		putative thioesterase
PA3226		putative hydrolase
PA3586		putative hydrolase
PA3942	*tesB*	putative acyl-CoA thioesterase I
PA4152		putative hydrolase
PA5384		putative lipolytic enzyme

Table S2: Pseudomonas Genome Database accession codes of hypothetical lipolytic enzymes in *P. aeruginosa* PAO1 genome.

PA0169	PA0847	PA1433	PA2072	PA2693	PA3177	PA3727	PA4792	PA5246
PA0201	PA0861	PA1486	PA2133	PA2771	PA3200	PA3741	PA4792	PA5295
PA0285	PA0924	PA1593	PA2168	PA2801	PA3241	PA3750	PA4830	PA5310
PA0290	PA0957	PA1594	PA2200	PA2858	PA3241	PA3825	PA4921	PA5329
PA0308	PA0968	PA1618	PA2218	PA2870	PA3251	PA3829	PA4929	PA5371
PA0338	PA0988	PA1640	PA2451	PA2871	PA3258	PA3910	PA4969	PA5442
PA0351	PA1107	PA1771	PA2540	PA3076	PA3301	PA3971	PA5017	PA5487
PA0449	PA1181	PA1851	PA2567	PA3087	PA3311	PA4093	PA5089	PA5519
PA0474	PA1204	PA1907	PA2580	PA3125	PA3339	PA4332	PA5185	
PA0575	PA1219	PA1962	PA2689	PA3130	PA3343	PA4440	PA5202	

Table S3: General data of phospho-lipolytic enzymes.

Name	PA number	Nucleotide length (bp)	Protein length (aa) mature / processed[a]	Protein weight (Da) mature / processed[a]
PlaK	1047	1176	392	42335
EstB	2098	927	309	33018
PldB	2155	1203	401	46545
TesA	2856	603	201 / 180	21038 / 18898
PlaB	2927	1332	444	49479
PlbF	2949	945	315 / 287	34837 / 31881
PlpD	3339	2184	728 / 710	80899 / 78898
EstC	3628	852	284	31275
PldC	4339	1077	359	41408
LipF	4921	921	307 / 187	33541 / 31505

[a] Protein after the cleavage of putative signal peptide

Table s4: Data collection and refinement statistic of the TesA.

Data collection[a]	
space group	C2
cell parameters	82.0 Å, 103.1 Å, 45.9 Å, 97.6˚
resolution range (Å)	40.6 – 1.7 (1.73 – 1.70)
number of unique reflections	40553 (1910)
Multiplicity	3.2 (3.2)
I/σ ratio	10.1 (2.3)
completeness (%)	97.9 (94.9)
R_{merge} (%)	7.6 (56.1)
Refinement statistic	
R factor (%)	24.2
R_{free} (%)	27.1
number of non-hydrogen atoms	
Protein	2642
Solvent	291
rmsd for bond distances (Å)	0.002
rmsd for bond angles (Å)	0.663

[a]The numbers in parentheses refer to data in highest resolution shell.
rmsd, root mean square deviation from the ideal bond distances and angels

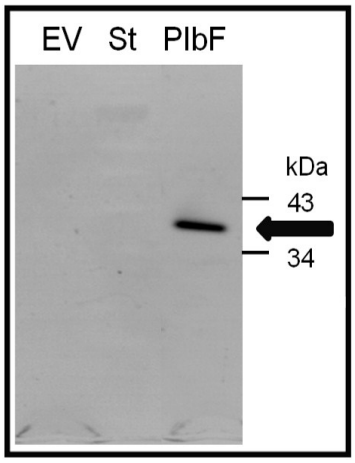


Figure S1: Zymographic detection of PlbF expressed in *P. aeruginosa* PA01. *P. aeruginosa* PA01 transformed with pBBR-PlbF (PlbF) and pBBR1mcs-3 (EV) was grown overnight under standard conditions without IPRG induction. On the SDS PAGE gel (12%) were analysed 0.15 U_{Cell} and zymographic detection was performed with MUB as a substrate (chapter s.s.s). St = standard protein markers which molecular weights in kDa are marked to the left. Black arrow indicates PlbF.

8. Curriculum vitae

Personal data:

Name:	Filip Kovačić
Date of birth:	11. June 1982
Place of birth:	Čakovec (Croatia)
Marital status:	married, father of two daughters
Address:	Lorsbecker Straße 52, 52428, Juelich

Education:

1996 – 2000	Secondary School Diploma, Chemical Technician, Mining and Chemical School, Varaždin (Croatia)
2000 – 2005	Diploma in Chemistry (Biochemistry), Division of Chemistry, Faculty of Science, University of Zagreb. Title: Inhibition of extracellular lipase from bacterium *Streptomyces rimosus* Supervisors: Prof. Dr. I. Weygand-Đurašević and Dr. sc. B. Kojić-Prodić

Working experience:

June 2005 - January 2006	Research assistant, Laboratory of Chemical and Biological Crystallography, Ruđer Bošković Institute, Zagreb, Croatia.
September 2009 - December 2009	Research assistant, Institute for Molecular enzyme technology at Research Center Juelich, Heinrich-Heine-University Duesseldorf.

Scholarships and Grants:

1997 & 1999	Third place on Croatian Competition of Young Chemists.
2000 - 2005	Stipend of Croatian Ministry of Science, Education and Sports.
2004	Rector's award at the University of Zagreb for the project entiteld "Preparation of polyelectrolyte multilayers on metal oxides particles"supervised byD. Kovačević.
2005	Short term fellowship of European Molecular Biology Organization for the project entiteld "Two novel GDSL-hydrolases from *Pseudomonas* aeruginosa PAO1" spervised by K.-E. Jaeger and S. Wilhelm.
2006	Marie Curie Early Stage Research Training fellowship in frame of Antibiotarget project (EU).

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