Molecular Analysis of the Chlamydial Anomaly:

Role of the cytoskeleton protein MreB, the serine hydroxymethyl transferase GlyA and the penicillin binding proteins from

Chlamydophila pneumoniae

Dissertation

Zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Alexandria, Ägypten

Bonn 2012

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät
der Rheinischen Friedrich-Wilhelms-Universität Bonn

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Tag der Promotion: 21.09.2012

Erscheinungsjahr: 2012

My beloved family

ACKNOWLEDGMENT

I would like to seize this opportunity to thank all those who have contributed, one way or another, to the completion of this work, which marks a very important milestone in my life. Without them and without their commitment and help, it would not have been possible for me to gain this great experience. It is therefore my pleasure to acknowledge the support and help of those who joined me throughout this journey.

First and foremost, I would like to express my profound gratitude to my supervisor Prof. Dr.-Hans-Georg Sahl for accepting me as a member in his research group. It was a great honor for me to join one of his research projects and I really want to thank him from my deep heart for accepting me to accomplish my Ph.D. research in his institute.

I would like also to express my deep gratitude to Prof. Dr. Gabriele König for her interest in my work and for kindly accepting to be my co-supervisor. My thank goes also to Prof. Dr. Harald Groß and Prof. Dr. Gabriele Bierbaum who honored me with their approval to be members of the doctoral committee.

I am indebted beyond measure to Dr. Beate Henrichfreise for giving me her inestimable advices and opinions during different stages of the research project, her continuous remarks and valuable suggestions for improving the overview and the presentation of this work. I would like to express my deep appreciation for outstanding help during writing and revising not only this thesis, but all scientific reports as well. I have really learned a lot from her.

At this point, I wish to express my sincere thanks to my dear colleagues at the Pharmaceutical Microbiology Unit, in particular Christina Pöllinger, Anna Klöckner, Stefania De Benedetti, Henrike Bühl and Christian Otten, for helping, encouraging and backing me up. Special thanks go to Stefania and Henrike for their kind concern and support. Thanks also extend to Inge Luhmer-Becker, Bedia Bugdaci and Karina Boltersdorf, whose supportive and friendly assistance made the last few years much easier.

My straightforward gratitude to the German Academic Exchange Service, DAAD (Deutscher kademischer Austausch Dienst) for selecting me as one of its scholarship

holders and for granting me the financial support to proceed my education in Germany, the country I have really loved. A special thank goes to Ms. Margret Leopold, from the DAAD office in Bonn, who was particularly helpful in all administrative stuff and supportive during the years of my scholarship. I gratefully acknowledge the graduate school BIOTECH-PHARMA for accepting me as collegiate Ph.D. student and for its well-organized, very beneficial workshops and lectures.

Very special thanks go to my family especially my precious mother for her heartily feelings, great supports and continuous prayers. A very special thank goes also to all my professors and colleagues in the Medical Research Institute, University of Alexandria, Egypt for their support, in particular Prof. Dr. Ola Abdel Kader.

Last but not least, an endless thank goes to my beloved wife Tahany Abdel Hameed for her support, her endless love, her encouragement and her patience with me during the hard days and also to my adorable daughter Hala for her cheerful smiles that could mitigate any sort of tiredness. I owe a lot to their support.

Ahmed Gaballah

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July 2012

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(v/v)	Volume/volume percent
(w/v)	Weight/volume percent
~	Approximately
°C	Degree Celsius
µg	Microgram
µL	Microliter
µM	Micromole
A22	S-(3.4-dichlorobenzyl)isothiourea
A260	Absorbance at 260 nm
A280	Absorbance at 280 nm
AMP	Adensoine monophosphate
ATP	Adenosine triphosphate
BACTH system	Bacterial adenylate cylcase two hybrid system
BSA	Bovine serum albumin
C_{55}-P	Undecaprenyl phosphate, Bactoprenol lipid carrier
cAMP	Cyclic adensoine monophosphate
CAP	Catabolite activator protein
Da	Dalton
D-ala-D-ala	D-alanyl-D-alanine
DAO	D-amino acid oxidase
dcw	division cell wall cluster
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EB	Elementary body
EDTA	Ethylene diamine tetracetic acid
EGTA	Ethylenglycol-bis(aminoehtylether)-tetraacetate
FAD	Flavin adenine dinucleotide

G	Gram
G	Relative centrifugation force to gravity
GlcNAC	N-acetylglucosamine
GTP	Guanidine triphosphate
His_6	Hexa-histidine tag
HMM-PBPs	High molecular mass penicllin binding proteins
HRP	Horse radish peroxidase
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
L	Liter
LB-medium	Luria-Bertani medium
Lipid I	C_{55}-PP-MurNAc-pentapeptide
Lipid II	C_{55}-PP-GlcNAc-MurNAc-pentapeptide
LMM-PBPs	Low molecular mass penicllin binding proteins
M	Molar
MCS	Multiple cloning site
m-DAP	Meso-dianinopimelic acid
MGT	Monofunctional transglycosylases
Min	Minute
Ml	Milliliter
mM	Millimolar
MurNAc	N-acetylmuramic acid
NF-κβ	nuclear factor κβ
Ni-NTA agarose	Nickel nitrotriacetic acid agarose
Nm	Nanometer
Nod	Nucleotide oligomerization domain
OD	Optical denisty
OD_{600}	Optical denisty at 600 nm
ONPG	O-nitrophenyl-β-D-galactoside
ORF	Open reading frame
P	Phosphate

PAGE	Polyacrylamide gel electrophoesis
PBP	Penicillin binding protein
PEP	Phosphoenolpyruvate
PG	Peptidoglycan
PMA	Phosphomolybdic acid
PP	Pyrophosphate
QBS	QuantaBlu substrate
RB	Reticulate body
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Round per minute
RT	Room temperature
S2d	N-benzoyl-D-alanylmercaptoacetic acid
SDS	Sodium dodecyl sulfate
Sec	Second
SHMT	Serine hydroxymethyl transferase
SOC medium	Super Optimal broth medium + glucose
TAE	Tris-acetate-EDTA buffer
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) diamine
UDP	Uridine diphosphate
UV	Ultraviolet
V	Volt

1 INTRODUCTION

1.1 BIOLOGY AND PHYLOGENY OF CHLAMYDIAE

Bacteria of the family *Chlamydiaceae* are obligate intracellular pathogens that infect both humans and animals causing ocular, respiratory and sexually transmitted diseases [1]. They are spherical eubacteria that resemble morphologically Gram-negative bacteria with inner and outer membranes [2]. *Chlamydiaceae* exhibit a unique biphasic developmental cycle which occurs within cytoplasmic vacuoles, known as inclusions, in the eukaryotic host cells [3].

Prior to the availability of molecular and genetic analysis, only four chlamydial species could be identified to compose the family *Chlamydiaceae* (Figure 1b). In taxonomy based on 16s RNA sequence analysis and DNA-DNA hybridization (Figure 1a), the family *Chlamydiaceae*, which includes all commonly known chlamydiae species, was classified as one of four families composing the order *Chlamydiales* [4, 5]. The *Chlamydiaceae* family consists of two distinguishable genera, *Chlamydia* and *Chlamydophila* [4, 6].

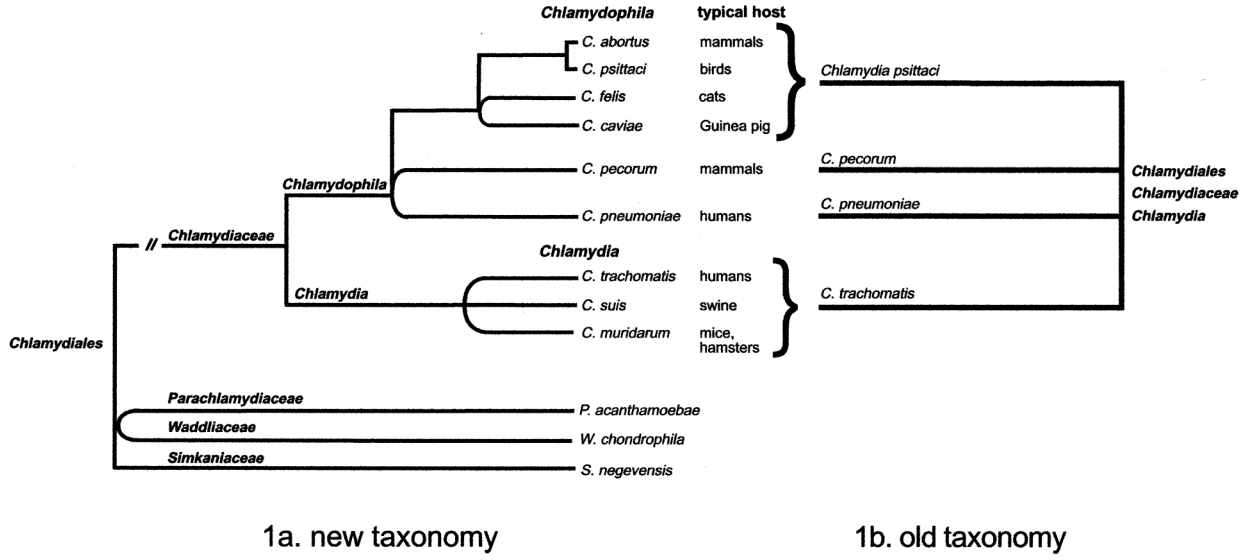

Figure 1: Phylogenetic tree of the order *Chlamydiales* [7].

1.1.1 Chlamydial infections

Three species of chlamydiae are known to cause infections in humans (Figure 1), *Chlamydia trachomatis*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci*.

C. trachomatis is the species responsible for about 90 million cases of sexually transmitted diseases worldwide. About one third of this number is contributed to the developed world [8]. Sexually transmitted *C. trachomatis* infection causes a pelvic inflammatory disease in women that may develop into scarring of the fallopian tubes causing infertility [9]. Additionally, *C. trachomatis* can cause ocular infections that are the main cause of blindness in developing countries [10, 11].

C. pneumoniae can cause respiratory tract infections. It is responsible for about 10% of community-acquired pneumonia cases and 5% of bronchitis and sinusitis cases. Infections are most common in children and most adults show serologic evidence of past infections [12].

C. psittaci is responsible for respiratory tract infections in birds. Infection can spread from birds to humans causing a rare type of pneumonia called psittacosis [13].

1.1.2 Life cycle of chlamydiae

The *Chlamydiales* exhibit a unique biphasic developmental cycle that distinguishes them from other intracellular bacteria [14]. Two forms of chlamydiae differentiate into each other during the life cycle, the elementary body (EB) and the reticulate body (RB) [15].

EBs represent the small (ca. 0.3 µm), spherical, extra-cellular form of chlamydiae [16, 17]. EBs are metabolically inactive but able to attach to and invade susceptible host cells spreading the infection. Although all attempts to detect peptidoglycan (PG) in EBs were unsuccessful [2], they are rigid and osmotically stable, which is a result of high degree of inter and intramolecular disulfide cross-linking of cysteine-rich proteins of the outer envelope including OmpA, OmcB, and OmcA [14, 18, 19].

In contrast to EBs, RBs are larger (ca. 1 µm), intracellular, and non-infectious chlamydiae [18]. RBs are metabolically active and can divide inside host cells by repeated

cycles of binary fission. RBs are osmotically instable and need to be protected within a membrane-bound-vacuole, known as inclusion, that provides an isotonic environment for chlamydiae [2].

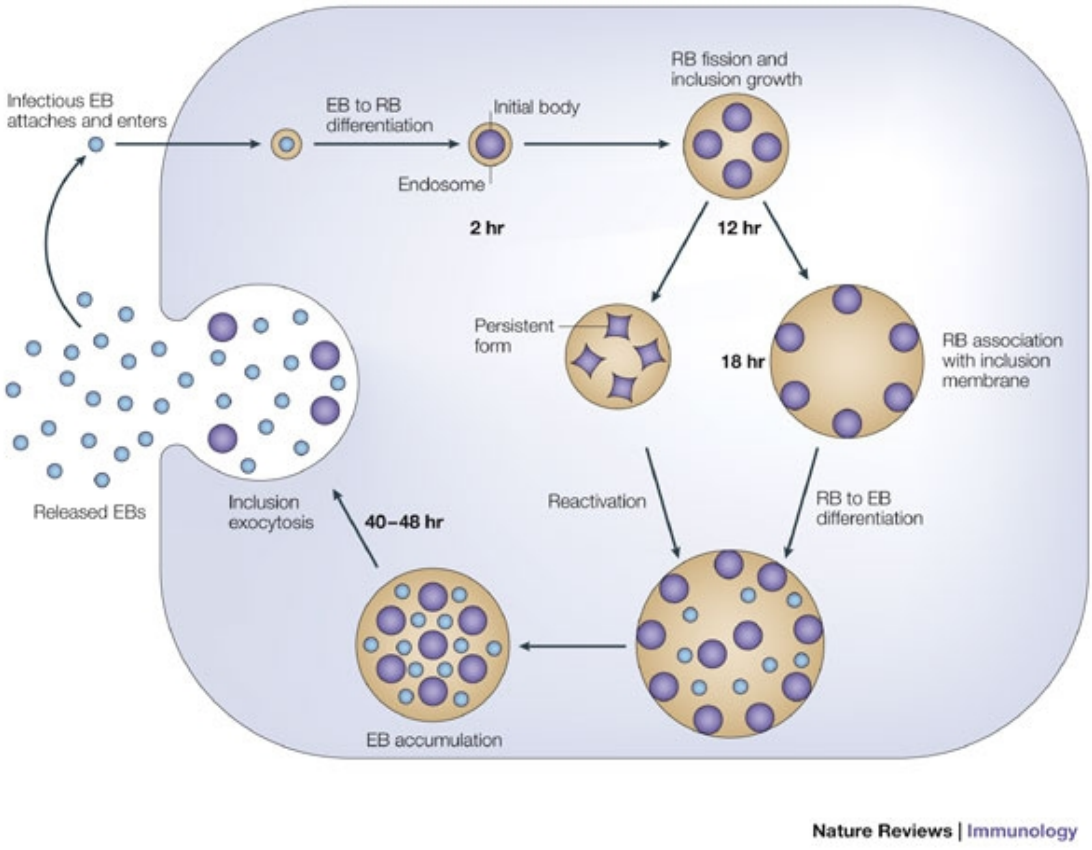

Figure 2: Developmental cycle of *C. trachomatis* [8].

EB = elementary bodies & RB = reticulate bodies

The developmental cycle (Figure 2) starts by attachment of the EBs to the susceptible host cells, which occurs in two stages. The first stage is reversible and takes place through electrostatic interactions between EBs and heparan sulfate receptors on host cells [20, 21]. The second stage is an irreversible binding that was demonstrated using mutagenized cell lines [22]. EBs entry into host cells is achieved via receptor-mediated-phagocytosis forming a membrane-bound-vacuole, termed inclusion [23]. Chlamydial inclusions do not fuse with endosomes and lysosomes, helping bacteria to bypass host immune system [14]. EBs within inclusions differentiate into the metabolically-active form, RBs, which divide by repeated cycles of binary fission. Finally, RBs differentiate back into infectious EBs, followed by lysis of host cells and release of EBs that infect adjacent cells

[18]. The chlamydial developmental cycle takes from 30 to 72 hours to complete according to the species [24].

1.1.3 Chlamydial anomaly

Being an obligate intracellular pathogen, chlamydiae have a very reduced genome size, roughly 1.1 Mb, compared to free living bacteria like *Escherichia coli* (*E. coli*) (~ 4.6 Mb) [25]. This feature reflects the evolutionary adaptation to host cell environment, which supports genome shrinkage. The genome of such organisms is thought to code only indispensable genes for their parasitic life [26]. Thus, chlamydiae do not need to keep cell walls to maintain osmotic stability in their isotonic intracellular environment, and all trials to detect PG failed to prove its presence [27].

Like all other cell wall-less bacteria, chlamydiae were not expected to be susceptible to antibiotics that target PG synthesis. Surprisingly, penicillin, and other antibiotics that target cell wall biosynthesis can interrupt the chlamydial developmental cycle [28-30]. In presence of penicillin, RBs can undergo 2 cycles of binary fission maximally. Prolonged exposure to penicillin leads to formation of aberrant enlarged RBs and the developmental cycle enters a persistent, reversible, non-infectious state (Figure 3) [24]. Moreover, genes encoding a nearly complete pathway for the biosynthesis of cell-wall precursor lipid II could be found in chlamydial genome [25, 27]. This paradoxical phenomenon is known as chlamydial anomaly [27, 29].

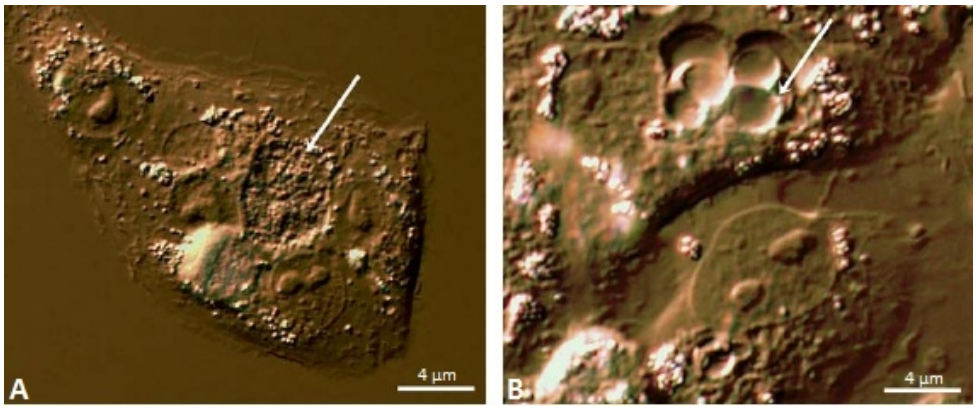

Figure 3: Interference contrast microscope images of inclusions after 24 hours of infection with *C. trachomatis* in absence (A) and presence (B) of penicillin [24].

The white arrow highlights the different morphology.

1.2 BACTERIAL CELL WALL BIOSYNTHESIS

The cell wall in bacteria is the element responsible for maintaining cell shape and cell integrity by providing mechanical strength and resisting osmotic challenges [31]. In both Gram-positive and Gram-negative bacteria, the sacculus surrounding the cytoplasmic membrane consists of cross-linked PG which is responsible for withstanding the high intracellular pressure [32, 33]. The PG synthesis is tightly connected to cell growth and cell division processes in bacteria [31].

PG biosynthesis is one of the main targets for antibiotic treatments as treating cells with β-lactam antibiotics or any substance that inhibit cell wall biosynthesis leads to cell lysis.

1.2.1 Chemical structure of peptidoglycan

Peptidoglycan, the main component of the cell wall sacculus, consists of linear glycan strands cross-linked with short peptides [34]. The glycan strands are built up of alternating units of N-acetylglucosamine (GlcNAC) and N-acetylmuramic acid (MurNAc) linked together by β-1,4-glycosidic bonds [33-36]. A stem peptide is attached to MurNAc by adding to its D-lactoyl group. This peptide consists mostly of L-Alanine-D-Glutamic acid-*meso*-diaminopimelic acid (or L-lysine)-D-alanyl-D-alanine [33, 36] (Figure 4). In the mature PG, the terminal D-alanine is removed during the process of cross-linking, which takes place between the carboxyl group of the D-alanine at position 4 and the amino group of the diamino-acid at position 3 of an adjacent glycan strand [36]. This cross link may be direct or via a short peptide bridge [31].

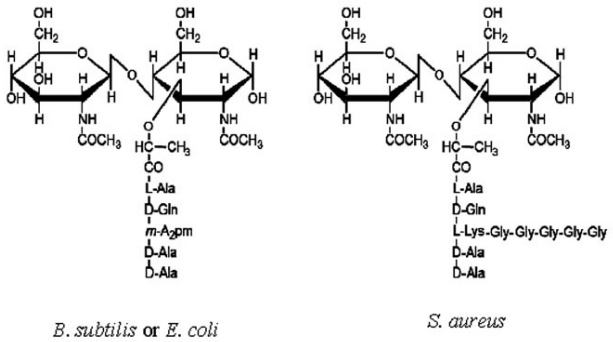

Figure 4: Chemical composition of the cell wall building block lipid II [37].

1.2.2 Peptidoglycan biosynthesis

The biosynthesis of PG occurs in three stages (Figure 5): the first one takes place in the cytoplasm, the second at the cytoplasmic membrane and the last stage occurs at the outer side of the cytoplasmic membrane [34, 38].

Biosynthesis starts by the transfer of enolpyruvate from phosphoenolpyruvate (PEP) to UDP-GlcNAc with the help of UDP-GlcNAc-enoylpyruvate transferase (MurA) followed by reduction of enolpyruvate moiety into D-lactate with help of the reductase enzyme (MurB) yielding UDP-MurNAc [39, 40]. To the D-lactoyl group of UDP-MurNAc, stepwise addition of amino acids takes place to form the stem peptide through Mur ligases (MurC, MurD, MurE and MurF). During this process the amino acids L-alanine (MurC), D-glutamic acid (MurD), dibasic amino acid (MurE) and dipeptide D-alanyl-D-alanine (D-ala-D-ala) are added sequentially yielding UDP-MurNAc-pentapeptide [31, 41]. The dibasic amino acid in the third position is either L-lysine (most Gram-positive bacteria) or meso-diaminopimelic acid (m-DAP) (most Gram-negative bacteria) [31, 36, 41]. The activity of the Mur ligases requires ATP hydrolysis and a divalent cation such as Mg^{2+} or Mn^{2+} [41]. The dipeptide D-ala-D-ala is synthesized by the activity of the D-ala-D-ala ligase enzyme Ddl and then ligated to the stem peptide by the activity of MurF enzyme [42, 43].

The second stage of cell wall biosynthesis starts by the transfer of UDP-MurNac-pentapeptide moiety to the membrane lipid carrier undecaprenyl phosphate (C_{55}-P or bactoprenol-P) yielding C_{55}-PP-MurNAc-pentapeptide (lipid I) [44]. This reversible reaction is catalyzed by UDP-MurNac-pentapeptide transferase (MraY), an integral membrane protein, which transfers the reaction from the cytoplasm to cell membrane [45, 46]. By the activity of a membrane associated protein, UDP-GlcNAc transferase (MurG), UDP-GlcNAc molecule is transferred to the hydroxyl group at position 4 of the muramoyl moiety of lipid I yielding the disaccharide building block C_{55}-PP-GlcNAc-MurNAc-pentapeptide (Lipid II) [47, 48]. At the end of this stage, lipid II is translocated across the cell membrane by the activity of the lipid II flippase (FtsW) [49, 50].

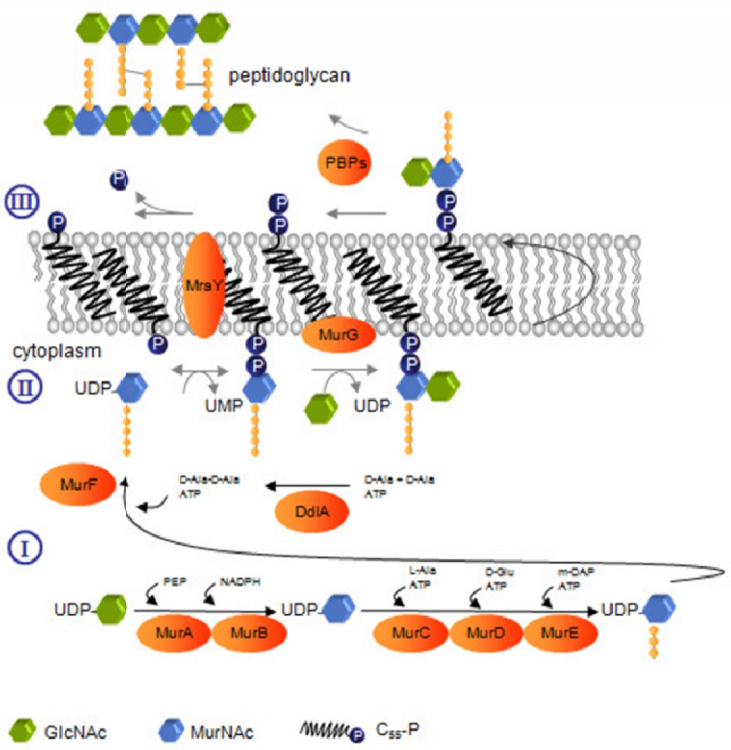

Figure 5: Schematic presentation of cell wall biosynthesis in *E. coli*.

(I) First stage of cell wall biosynthesis in the cytoplasm starting from UDP-Glc-NAc resulting in UDP-Mur-NAc-pentapeptide. (II) Second stage of cell wall biosynthesis at the cytoplasmic membrane leading to the synthesis of lipid I and lipid II. (III) Last stage of cell wall biosynthesis, incorporation of lipid II in cell wall and its recycling. (Scheme of Dr. Tanja Schneider)

The last stage of cell wall biosynthesis involves the polymerization and incorporation of the newly synthesized lipid II molecules into the growing PG. These reactions are catalyzed mainly by a group of enzymes called penicillin binding proteins (PBPs) which activate the transglycosylation and transpeptidation reactions to polymerize the glycan chains and to cross-link the peptides respectively [31, 37, 51].

At the end, the released lipid carrier C_{55}-PP is reactivated by hydrolysis of the pyrophosphate group and the release of the active lipid carrier C_{55}-P by the enzyme C_{55}-pyrophosphate phosphatase [52-54].

1.3 BACTERIAL CYTOSKELETON PROTEINS

Comparable to eukaryotes, bacteria use cytoskeletal proteins, such as actin and tubulin homologs, to control many cellular activities and processes, including cell morphogenesis, cell division, DNA partitioning, and cell motility [55]. The bacterial

cytoskeleton consists of filamentous structures that are composed of polymers of single protein molecules. These proteins are capable of self-assembling into polymeric filaments *in vitro* in the presence of conditions that favor their polymerization [56]. Four types of cytoskeletal proteins are well established in rod shaped bacteria like *E. coli*, *Bacillus subtilis* and *Caulobacter crescentus*. Three of these four types have homology to eukaryotic actin, tubulin and intermediate filament proteins. The fourth type, MinD protein, has no clear homologs in eukaryotic cells [57].

1.3.1 The bacterial actin homolog MreB

MreB, the actin-related cytoskeletal protein, plays an important role in regulation of cell shape, cell polarity and chromosome segregation in bacteria [56, 58]. It is widely distributed in bacteria with complex morphology (rods, vibrios and spirilla) but absent in most bacteria with spherical morphology [57, 59]. Some bacteria like *E. coli* possess a single MreB protein, while others (mostly Gram-positive) contain multiple MreB copies like *B. subtilis* which has MreB, Mbl and MreBH [56, 60]. *mreB*-knockout mutants and MreB depleted cells lose their typical shape and generate enlarged cells with major morphological defects [61-63].

MreB and actin are members of a superfamily of proteins, which includes Hsp70, cell division protein FtsA and sugar kinases. While all members of this superfamily have very little amino acid sequence identity, they share the characteristic actin core and its three dimensional fold which consists of α and β domains folding around a central core, the nucleotide-binding pocket [58, 59]. Among all these proteins MreB and actin are the most closely related in overall size [64]. With the exception of MreB and actin, the activities of these protein superfamily members are totally divergent and have little to do with cytoskeleton, but they are capable of binding and hydrolyzing ATP at their equivalent ATPase domain [64].

MreB is considered to organize into helical filaments that coil around the rod shaped cells under the surface of the cytoplasmic membrane [56]. Nevertheless, results from a recent study using total internal reflection fluorescence microscopy indicate that MreB might assemble into small patches in *B. subtilis, E. coli* and *C. crescentus* [65]. *In vitro,* MreB

proteins self-assemble into long polymeric filaments and the filaments interact to form bundles of solid-like structures with high elasticity that is thought to play a role in supporting cell shape. However, the highly dynamic nature of MreB and its continuous redistribution within cells suggest that rigidity of MreB polymers might not be essential for supporting cell shape [56]. This idea was supported by the effect of A22 (S-(3,4-dichlorobenzyl)isothiourea), a substance that inhibits MreB polymerization [66, 67], in rod-like shaped *C. crescentus*. Treating *C. crescentus* with A22 causes shape alteration very late in comparison to the disappearance of helical MreB structures [68]. It is likely that MreB maintains the shape of rod-shaped bacteria by orienting the PG biosynthetic enzymes to tether incorporation of cell wall precursors into the side-wall during the longitudinal growth [56, 59]. MreB filaments are thought to recruit and functionally organize enzymes involved in cell wall biosynthesis including soluble (MurB, MurC, MurE and MurF) and membrane (MraY and MurG) proteins [65]. The MreB organized machinery is connected to the PBP2 catalyzed lipid II precursor polymerization reactions on the outside through a membrane-spanning complex consisting of MreC, MreD and RodA [32, 61, 65, 69] (Figure 6A).

MreB filaments are flexible and highly dynamic structures that move continuously through the cell [59]. During cell divison, MreB filaments are rearranged into two circumferential rings that flank the cytokinetic FtsZ ring (section 1.3.2). The cytoskeletal MreB rings are suggested to be involved in the early cell division process, preseptal elongation and segregation of the cytoskeleton and show interactions with MreC, MreD and RodA as well [32, 70] (Figure 6B).

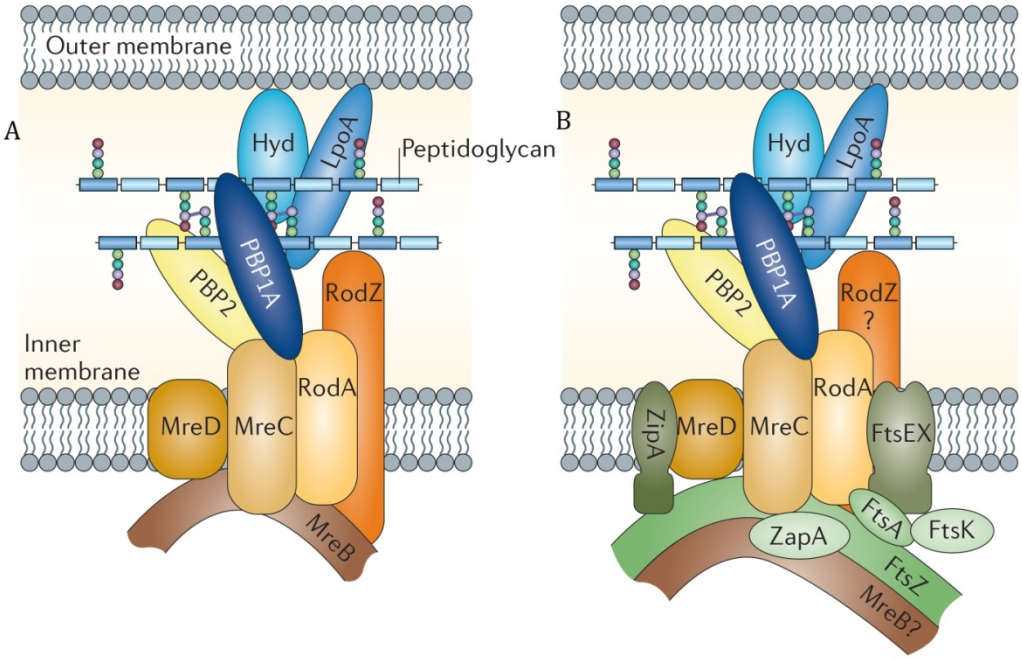

Figure 6: Schematic presentation of the organization of cytoskeleton-associated cell wall synthesizing proteins in *E. coli.*

(A) MreB and membrane associated proteins position PBPs during cell wall elongation. (B) Circumferential ring MreB flanks FtsZ ring which recruits enzymes for peptidoglycan synthesis to the septum during preseptal elongation [32].

1.3.2 The bacterial tubulin homolog (FtsZ)

Despite enormous progress in the last decade towards the identification of proteins involved in cell division and their mode of assembly, the biochemical functions of several division proteins are still poorly understood [71, 72]. Currently, the following stages of bacterial cell division are recognized: the tubulin homolog FtsZ assembles into an annular structure (Z-ring) at the site of division and initiates cell division by attracting a set of proteins to form a cytoplasmic apparatus linked to the membrane; then additional elements build an extracellular connector; and finally, proteins (e.g. transpeptidase FtsI (PBP3) and flippase FtsW) involved in the incorporation of septal peptidoglycan assemble (Figure 7). The process ends with constriction of the septum [71, 73]. According to the results from the localization of green fluorescent protein fusions or the immunostaining of the proteins in cell division mutants, the division proteins are recruited into the divisome machinery in a sequential and almost linear pathway [74, 75].

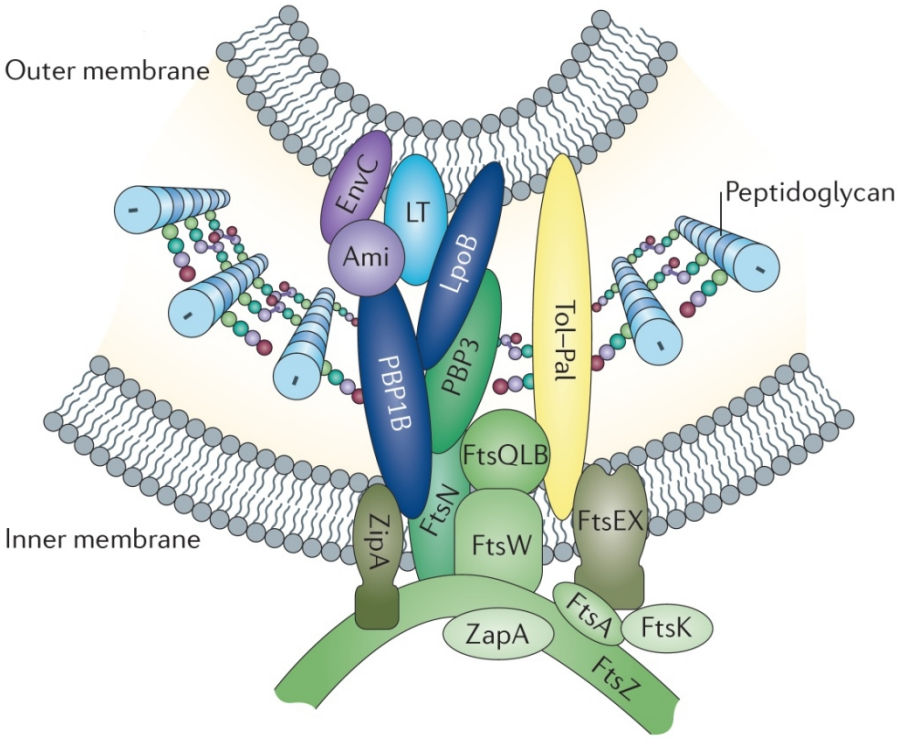

Figure 7: Schematic presentation of cell division machinery in *E. coli* [32].

During cell division, FtsZ protein builds up the Z-ring, which recruits other proteins that compose the divisome machinery.

The bacterial tubulin homolog FtsZ is an important cell division protein and almost ubiquitously distributed in bacteria, archae and eukaryotic organelles [56]. Like tubulin, FtsZ binds and hydrolyze GTP and its polymerization is GTP dependant [76, 77].

1.4 CYTOSKELETON AND CELL DIVISION PROTEINS IN CHLAMYDIAE

Chlamydiae were expected to harbor the essential cytokinesis protein FtsZ and lack the rod-shape maintaining protein MreB. Surprisingly, chlamydiae lack the highly conserved FtsZ but harbor, despite their spherical shape, MreB [25].

For cell division, a rudimentary set of divisome proteins lacking the almost ubiquitous central organizer FtsZ, but including FtsW, FtsI, FtsH, FtsK and FtsY has been found to be encoded by chlamydial genomes [78].

1.5 LIPID II BIOSYNTHESIS IN CHLAMYDIAE

The complete genome analysis of chlamydiae at the end of the 1990s [25] revealed a nearly complete pathway for the synthesis of lipid II in chlamydiae [27, 79-81] (Figure 9). Moreover, the nucleotide oligomerization domains NodI and NodII can recognize intracellular *C. pneumoniae* and subsequently mediate activation of the nuclear factor κβ (NF-κβ) playing a key role in regulating the immune response to infection [82, 83]. Nod receptors are members of the human cytosolic innate immune pattern recognition receptors sensing bacterial cell wall fragments [84-86], NodI has been described to recognize lipid II containing m-DAP [85, 87] whereas NodII has been described to recognize MurNAc-dipeptide fragments of cell wall [88, 89].

In all Gram-negative bacteria, the division cell wall (dcw) cluster is highly conserved. On the other hand, this gene cluster in the intracellular chlamydiae shows some unique differences such as the fusion of *ddl* to *murC* and the localization of the *murE* gene outside the dcw cluster (Figure 8).[2]

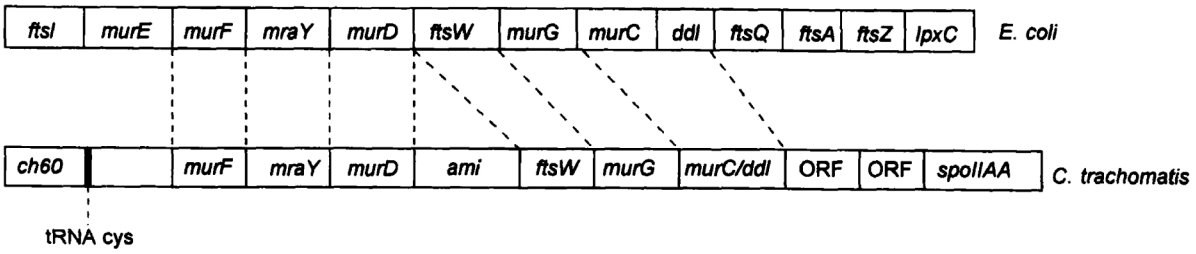

Figure 8: Comparison of the dcw cluster of *E. coli* and *C. trachomatis* [27].

ORF= open reading frame encoding product of unknown function.

So far, few studies have been conducted to investigate whether chlamydial genes of the PG biosynthesis pathway encode functional enzymes. These studies proved the functionality of MurA, Murc-Ddl, MurE and MurF, enzymes involved in the cytoplasmic synthesis steps of the PG precursor UDP-MurNAc-pentapeptide [90-94].

Additionally, the functionality of MraY and MurG, enzymes responsible respectively for lipid I and lipid II biosynthesis at the cytoplasmic membrane, has been demonstrated *in vitro* [95]. Concerning the last stage of cell wall biosynthesis in chlamydiae at the outer surface of the cytoplasmic membrane, the chlamydial genomes lack glycosyltransferases

that catalyze the formation of linear glycan chains of PG [2]. However, polymerization of PG precursor could be achieved through the transpeptidase activity of the monofunctional PBP2 and PBP3 encoded in chlamydial genomes [78]. Moreover, Chlamydiae lack endopeptidases which allow remodeling of the wall PG [2]. Cleavage of lipid II by the activity of the chlamydial amidase AmiA could be demonstrated *in vitro* [diploma thesis of Anna Klöckner].

The recycling of lipid carrier C_{55}-PP at the end of the biosynthetic pathway is still unclear. Pyrophosphorylases that can activate the lipid carrier by dephosphorylation are not present in chlamydial genomes.

The exact composition of the chlamydial lipid II pentapeptide side chain is still a matter of debate. It was shown that chlamydiae are able to synthesize m-DAP via an aminotransferase pathway through the activity of CT390 and DapF proteins [96]. The chlamydial genomes do not encode the racemases Alr and MurI, enzymes that catalyze the interconversion of L- and D- optical isomers of alanine and glutamic acid respectively [2]. This finding suggests that D-amino acids may not be part of chlamydial lipid II. However, the functionalities of Ddl domain of the MurC-Ddl fusion protein as D-ala-D-ala ligase [94] and MurF as D-alanyl-D-alanine adding enzyme [92] suggest that D-alanine should be a part of the chlamydial lipid II peptide side-chain. Indeed, no clear source for D-alanine could be proven until now and the hypothesis of D-alanine uptake from the host cell is highly questioned [2]. However, Patin *et al.* [92] postulated a peptide side chain in chlamydiae composed of L-alanine/L-serine/glycine-γ-D-glutamic acid-m-DAP-D-alanyl-D-alanine.

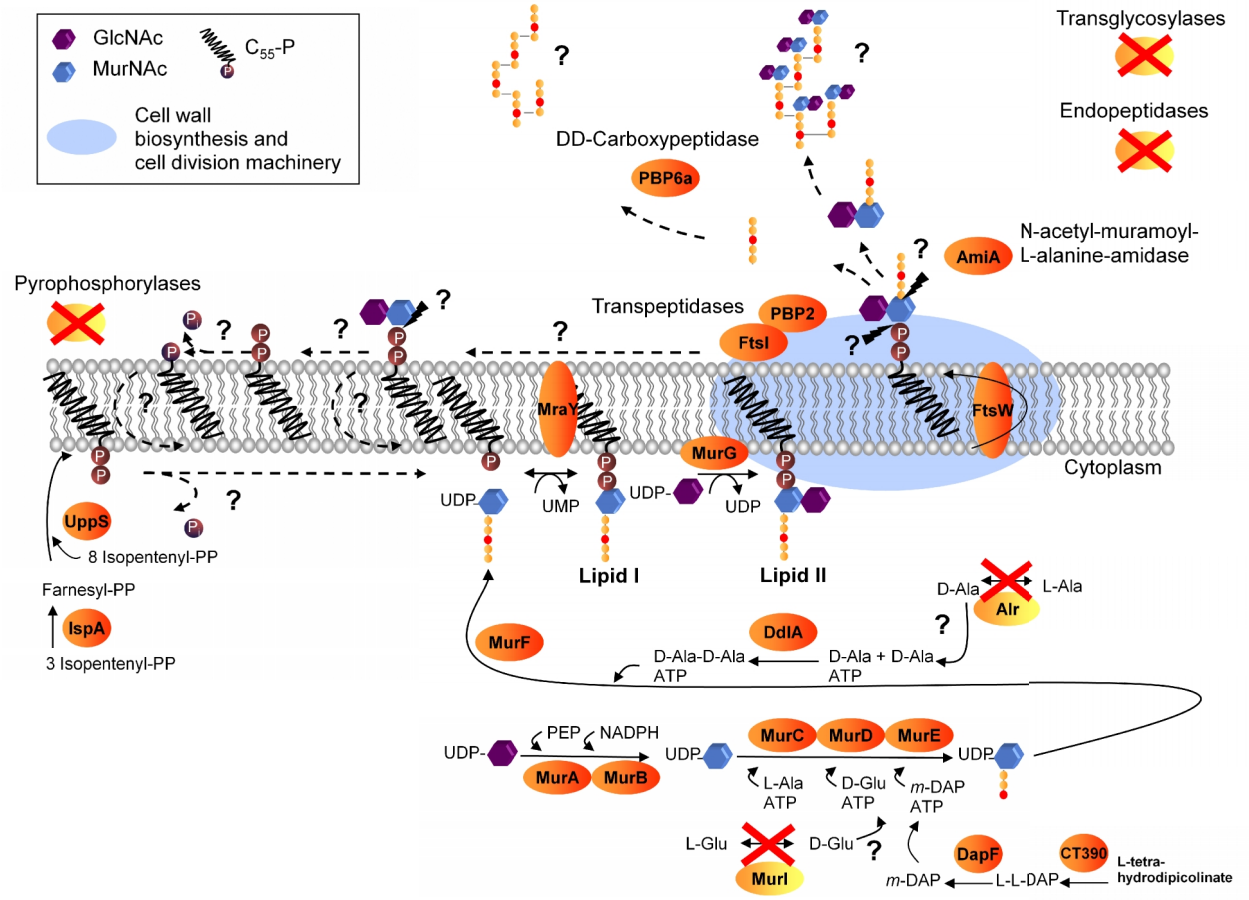

Figure 9: Hypothetical model for the lipid II biosynthesis pathway in chlamydiae (modified from [95]).

Genes not found in the genome are highlighted in red crosses. Question marks and dashed arrows indicate steps that need to be elucidated.

1.6 SERINE HYDROXYMETHYL TRANSFERASES (SHMTS)

Serine hydroxymethyl transferases (SHMTs) are enzymes that catalyze the reversible interconversion of serine and glycine with tetrahydrofolate serving as one carbon acceptor [97, 98]. SHMTs are widely distributed in mammalians, plants and prokaryotes [99]. The enzymes catalyze several reactions characteristic of pyridoxal-P enzymes. Noteworthy, rabbit liver cytosolic and *E. coli* SHMTs have been demonstrated to catalyze the racemization reaction of D- and L-alanine *in vitro* [98]. In a recent study, a D-alanine *E. coli* auxotrophic strain was constructed by deletion of *alr* and *dadX*, coding for alanine racemases. Proteome analysis of this auxotrophic strain and its phenotypic prototrophic revertant strain revealed that the SHMTs GlyA was one of the significantly

upregulated proteins in the phenotypic revertant together with other proteins involved in methionine synthesis [100].

Interestingly, the very-reduced chlamydial genome harbors a *glyA* gene. Additionally, phylogentic analysis of the conserved inserts in chlamydial *glyA* provides evidence that the gene has undergone lateral exchange with other free living actinobacteria [101] indicating that this gene is of great necessity for such intracellular pathogens.

1.7 COORDINATION OF CELL-WALL BIOSYNTHESIS AND CELL DIVISION IN PROKARYOTES

In rod shaped bacteria such as *E. coli* or *B. subtilis*, cell wall biosynthesis is a continuous process during cell elongation and cell division. On the other hand, round-shaped bacteria show cell wall biosynthesis only at the division septum [37]. At the septum, cell wall biosynthesis and cell division processes are both driven by partially overlapping machineries. The activity of both machineries needs to be tightly coordinated to maintain cell integrity. A recent finding suggests that peptidoglycan biosynthesis at the cell wall is driven by a multi-enzyme complex that is directly connected to the cell division machinery. For example, FtsI (PBP3), transpeptidase incorporating lipid II at the septum (section 1.2.2) is a crucial member of the divisome machinery.

1.8 ROLE OF THE LIPID II BIOSYNTHESIS PATHWAY IN CELL DIVISION

For intracellular bacteria, such as chlamydiae, the highly-energy consuming biosynthesis of PG appears to be superfluous. Nevertheless, several findings discussed in section 1.5 clearly confirm that the biosynthesis pathway of cell wall building block lipid II is conserved in the wall-less chlamydiae suggesting another essential role of the precursor besides cell wall biosynthesis in bacteria.

Recently, it was hypothesized that conservation of lipid II biosynthesis in intracellular bacteria reflects an intimate molecular linkage of cell division and cell wall biosynthesis and thus a vital role of the completed cell wall building block in cell division

[95] (Figure 10). Apparently, an entire cycle of lipid II biosynthesis and processing, including translocation of the precursor to the outside and recycling of the membrane carrier C_{55}-P is required for coordinated function of the cell division machinery [95].

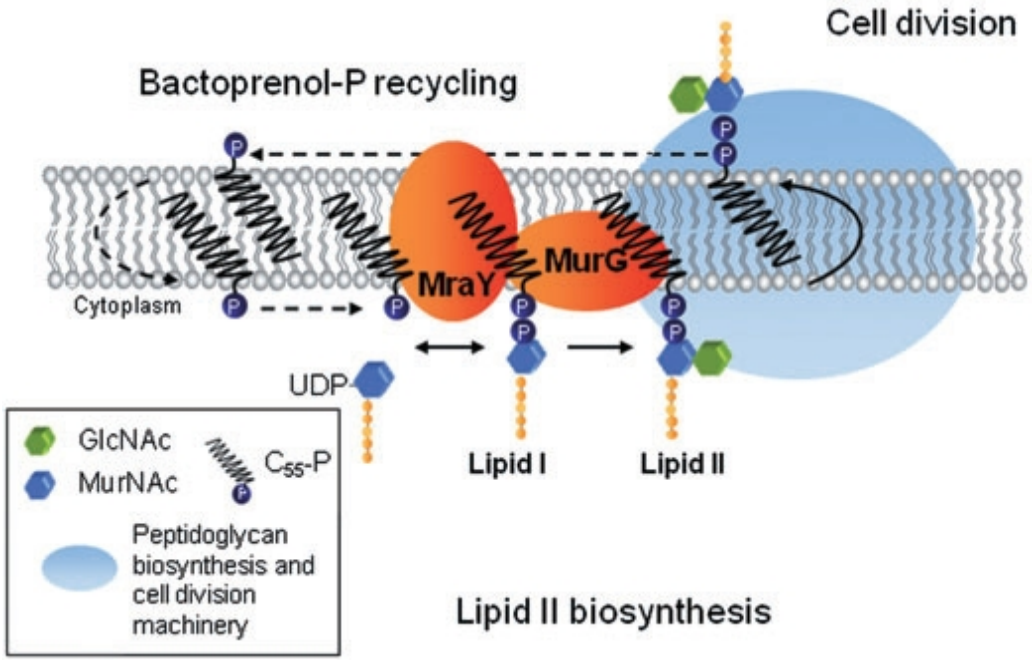

Figure 10: Proposed model for a link between lipid II biosynthesis pathway and cell division [95]

Lipid II is synthesized via a fully conserved pathway and then processed by a rudimentary cell wall biosynthesis-cell division machinery (Ftsw, FtsH, FtsK, FtsY). This machinery may contain MraY, MurG, PBPs and division proteins. Lipid II translocation to the outer side of the cell membrane and lipid carrier C_{55}-P recycling are required for coordinated function of the divisome machinery [95].

This hypothesis is strongly supported by the results of a study on the division of chloroplasts in mosses. Chloroplasts with disrupted MurE were unable to divide and grew in the form of macrochloroplasts due to inability to synthesize a functional lipid II [102]. A connection between lipid II biosynthesis and cell division in chlamydiae is also confirmed by aspects of the chlamydial anomaly. Penicillin does not show its normal bactericidal effect in chlamydiae, but stops the developmental cycle at the stage of RB division leading to formation of atypically enlarged non-dividing RBs within expanded inclusions [24].

1.9 PENICILLIN BINDING PROTEINS

Penicillin binding proteins (PBPs) are membrane bound enzymes, which are essential for the final steps of PG biosynthesis [37, 103]. Four different physiological functions are possibly assigned to PBPs (Figure 11). (i) Transglycosylation, in which PBPs catalyze the polymerization of disaccharide units forming glycan strands, (ii) transpeptidation, in which PBPs catalyze the cross-linking of the peptide stem, (iii) DD-carboxypeptidation, in which PBPs hydrolyze the terminal D-alanine of the peptide stem limiting the extent of PG cross-linking, or finally (iv) endopeptidation, which is the reverse activity of transpeptidation where PBPs hydrolyze the peptide bond cross-linking the glycan strands [103]. PBPs belong to a larger family of proteins known as acyl serine transferases, which includes both PBPs and β-lactamases [104].

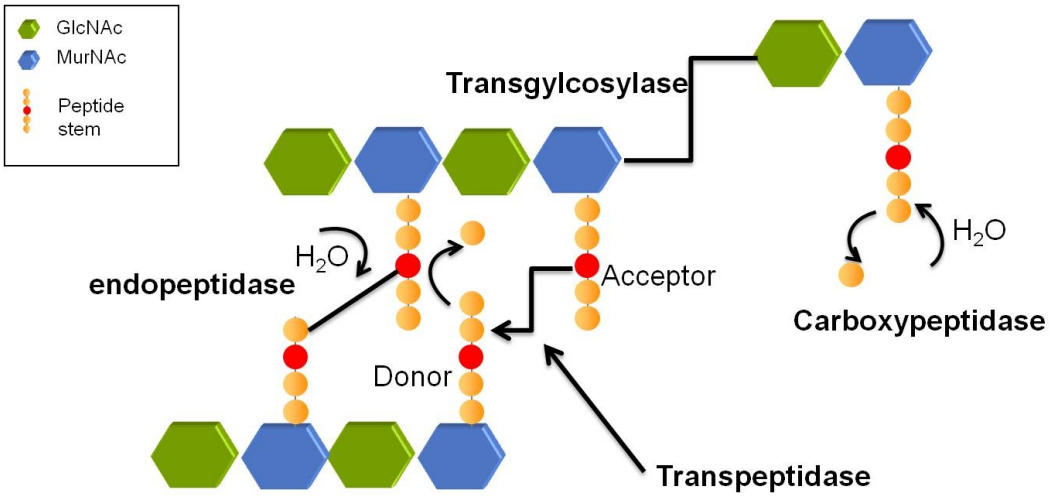

Figure 11: Schematic presentation of PBPs activities.

According to their molecular mass, PBPs can be classified into high molecular mass (HMM) PBPs and low molecular mass (LMM) PBPs [37, 103]. HMM-PBPs are composed of two modules anchored to the outer surface of the cytoplasmic membrane [37] and are responsible for peptidoglycan polymerization into growing cell wall [105, 106]. The HMM-PBPs are further classified according to the structure and activity of their N-terminal domain into class A and class B HMM-PBPs. While the C-terminal domain of all HMM-PBPs shows transpeptidase activity, the N-terminal domain of class A provides transglycosylase activity [103]. In class B, the N-terminal domain is believed to be involved in maintaining

cell morphology by providing a recognition site for interaction with other proteins during the cell cycles [33, 107, 108]. Monofunctional transglycosylases (MGTs), with similarity to the N-terminal domain of class A HMM-PBPs, are also found in some bacteria [109-111]. LMM-PBPs are monofunctional DD-carboxypeptidases or endopeptidases [104, 112].

Bacteria have a variable number of PBPs which are historically numbered according to their migration on SDS-PAGE [103]. *E. coli* possesses twelve PBPs, three (PBP1a, PBP1b and PBP1c) are class A HMM-PBPs, two (PBP2 and PBP3) are class B HMM-PBPs, and seven LMM-PBPs exhibit DD-carboxypeptidase or endopeptidase activities [37, 103]. Although the biochemical activities of all these PBPs are known, their exact role within the cell is still not completely determined. *E. coli* can survive even with inactivation of eight of these twelve PBPs [113]. PBP1a and PBP1b are the major transpeptidases-transglycosylases in *E. coli*. Deletion of one of them is not lethal but inactivation of both results in autolysis [114-116]. PBP2 is essential for cell elongation and its loss results in spherically-shaped cells instead of rods [117]. By contrast, the second monofunctional transpeptidase PBP3 is involved in cell division and its absence causes cell filamentation [118, 119]. Only PBP2 and PBP3 in combination with either PBP1a or PBP1b are necessary to maintain the phenotype of wild-type *E. coli* [113].

1.10 PENICILLIN BINDING PROTEINS IN CHLAMYDIAE

The sensitivity of chlamydiae to penicillin and other β-lactams has been related to the presence of PBPs [29, 120], and three PBPs were detected in lysated *C. trachomatis* using direct labeling with radioactive penicillin [120]. In accordance, three open reading frames encoding proteins with homology to PBPs have been found in chlamydial genomes [78, 112]. Amino acid sequence analysis of the three chlamydial PBPs indicates that two of them are class B HMM-PBPs equivalent to PBP2 and PBP3 from *E. coli* with the monofunctional transpeptidases and the third PBP can be classified under LMM-PBPs with greatest similarity to the carboxypeptidase PBP6a from *E. coli* [2, 27, 121].

2 AIM OF THE PROJECT

2.1 FUNCTIONAL ANALYSIS OF CHLAMYDIAL MREB

Based on the hypothesis that lipid II biosynthesis may have to be maintained for functional cell division, MreB could be involved in coordinating cell division via tethering the biosynthesis of lipid II to the chlamydial septum. In this project, it was investigated whether chlamydial MreB (i) polymerizes *in vitro*, (ii) hydrolyzes nucleotides and (iii) interacts with proteins involved in lipid II biosynthesis, such as MurF, MraY and MurG.

2.2 STUDIES ON THE STRUCTURE OF CHLAMYDIAL LIPID II

PBPs bind their substrate via a D-alanyl-D-alanine (D-ala-D-ala) residue at the C-terminus of the pentapeptide side chain of lipid II. To investigate the presence of such a target in chlamydiae, I will check the capability of chlamydiae to synthesize D-alanine. *Chlamydiaceae* lack the alanine racemase enzyme responsible for the synthesis of D-alanine in all other eubacteria but encode the serine hydroxymethyltransferase (SHMT) GlyA that has been shown to catalyze racemization of D- and L- alanine. In order to test if GlyA activity can provide a way to synthesize D-alanine in *Chlamydiaceae*, the racemase activity of chlamydial GlyA was investigated *in vitro*.

2.3 FUNCTIONAL ANALYSIS OF PBPS FROM CHLAMYDIAE

Chlamydiae harbor genes encoding three PBPs which resemble PBP2, 3, and 6a from *E. coli*. PBP2 and PBP3 from *E. coli* are mono-functional transpeptidases, which cross-link glycan chains via peptide bridges in free-living bacteria. PBP6a from *E. coli* shows DD-carboxypeptidase activity responsible for the removal of terminal D-alanine of the peptide side chain blocking transpeptidation. Chlamydial PBPs might be essential for the processing of lipid II at the septum catalyzing the release of the membrane carrier bactoprenol. To analyze the functionality of PBPs in chlamydiae, the three proteins were cloned, over-produced and purified, and tests were established to detect their activities *in vitro*.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Reagents and chemicals

Reagents and chemicals utilized in this work are listed in Table 1 and were of analytical grade or better.

Table 1: Chemicals and materials

Company	Chemical name
Agilent Technologies/Stratagene (Waldbronn, Germany)	QuickChange Lightning Site-Directed Mutagenesis Kit
Baker (Griesheim, Germany)	Chloroform, methanol, water (HPLC-grade)
Biorad (München, Germany)	Bradford protein assay
Fermentas/Thermo Sientific (St. Leon-Rot, Germany)	GeneRuler 1 kb DNA ladder, PageBlue, Pierce protein gel (4-20% SDS, 12 und 17 Well), Pierce 4x LDS sample buffer, Top vision agarose, Tris-Hepes-SDS-running buffer, Zeba Desalt Spin columns
Fulka (Steinheim, Germany)	Imidazole, Bovine serum albumin (BSA),Triton X-100
Hannah Instruments (Kehl am Rhein, Germany)	Calibration solutions pH 4,1/ pH7,1
Larodan Fine Chemicals (Malmö, Sweden)	Undecaprenylphosphat (C55-P)
Macherey-Nagel (Düren, Germany)	Ni^{2+}-NTA-Agarose
Merck (Darmstadt, Germany)	Agar-Agar, ammonium carbonate (NH_4CO_3), ammonium sulfate $(NH_4)_2SO_4$, 1-butanol, disodium hydrogen phosphate·dihydrat (Na_2HPO_4 2 H_2O), acetic acid glucose monohydrate, yeast extract, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethansulfonic acid (Hepes), potassium chloride (KCl), potassium hydroxide, silica gel plates 0,25 nm, magnesium sulfate ($MgSO_4$), Sodium chloride (NaCl), sodium dihydrogen phosphate dihydrat (NaH_2PO_4 2 H_2O).EGTA, MreB perturbing substance A22, perchloric

Company	Chemical name
	acid
MP Biomedicals (Illkirch, GmbH)	Glycerol, tryptone
New England Biolabs (Ipswich, USA)	Desoxynucleotide (dNTPs), physion DNA polymerase, protein marker 7702S, restriction enzymes
Operon (Ebersberg, Germany)	Oligonucleotide (primers)
Pharmacia Biotech (Düsseldorf, Germany)	Dithiothreitol (DTT)
Qiagen (Hilden, Germany)	QIAprep Spin Miniprep kit, polypropylen columns (1 ml), Minelute PCR purification Kit
Roche (Basel, CH)	T4-DNA-Ligase, 10x ligation buffer
Roth (Kalsruhe, Germany)	Ethidium bromide-solution, sodium dodecyl sulfate (SDS)-pellets
Sigma-Aldrich (Steinheim, Germany)	Adenosine triphosphate (ATP), ammonium hydroxide, ampicillin sodium, D-alanyl-D-alanine, D-glutamic acid, dimethyl sulfoxide (DMSO), desoxyribonuclease (DNase), ferrous (II)-sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), isopropyl-β-D-thiogalactopyranosid (IPTG), kanamycin, L-lysine, lysozyme, magnesiumchloride hexahydrate ($MgCl_2 \cdot 6H2O$), meso-diaminopimelic acid (m-DAP), N-dodecyl-β-maltoside (DDM), nicotinamide-adenine-dinucleotide (NADPH), sodium lauroylsarcosin, o-nitrophenyl-β-D-galactopyranosid (ONPG), perchloric acid, ribonuclease A (RNase A), thiamine hydrochlorid, Tris base, Tris hydrochlorid, uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), zinc sulfate ($ZnSO_4$), α-chymotrypsin, β-mercaptoethanol, PG sacculi from *B. Subtilis*

3.1.2 Utensils and laboratory equipments

All equipments, labware and consumables used in this work, together with their respective manufacturers and suppliers, are listed in Table 2.

Table 2: Utensils and laboratory equipments

Manufacturers	Equipments
Abimed GmbH (Langenfeld, Germany)	Automatic pipette Gilson Pipetman P2, P20, P100, P200, P1000, P5000
AEG (Berlin, Germany)	Microwave
Agfa-Gevaert N.V.(Mortsel, Belgium)	Agfa scanner SnapScan™ e40
Barnstead International (Dubuque, Iowa, USA)	Deionizer, Easy Pure LF®
Beckman Coulter Inc. (Fullerton, USA)	Centrifuge: J2-21
Bibby Sterlin Ltd. (Stone Staffordshire, GB)	Rotary evaporator RE 100
BioRad Laboratories (München, Germany)	Thermocycler iCycler, Molecular Imager GelDoc XR+, Image Lab Software Version 2.0.1
Branson (Danbury, USA)	Ultrasonic bath Bransonic 1510 E-MT
Braun Biotech International (Melsungen, Germany)	Centrifuge Sigma 2K 15, shaking water bath CERTOMAT® H, 1 ml syringes, OMNIFIX®
Dr. Hielscher Ultrasonics GmbH (Teltow, Germany)	Ultrasonic homogenizer UP50H
Eppendorf (Hamburg, D)	MiniSpin® microfuge
Gesellschaft für Labortechnik (GFL, Burgwedel, D)	Shaking water bath 1083
Glaswerk Wertheim (Wertheim, Germany)	Exicator
Grant Instruments (Cambridge, UK)	Heating block QBH2
Hannah Instruments (Kehl am Rhein, Germany)	HI 221 Microprocessor pH-Meter
Harry Gestigkeit GmbH (Düsseldorf, Germany)	Precision hot plate
Heraeus Holding (Hanau, Germany)	37°C incubator, Rotator incubator, Biofuge 28 RS, rotator incubator
Heraeus Sepatech GmbH (Osterode, Germany)	Centrifuge, Biofuge 28RS, rotors 3751,

Manufacturers	Equipments
Implen (München, Germany)	Nanophotometer™ 7122v1.6.1
Invitrogen (Carlsbad, USA)	Novex Mini Cell (SDS-Gel-chamber)
Julabo Labortechnik GmbH (Selbach, Germany)	Mini water bath Julabo MWB
Martin Christ (Osterode, Germany)	Freeze-dryer
PALL GmbH (Dreieich, Germany)	Sterile Acrodisc® syringe filters, 0.2 µm
Paul Hartmann AG (Heidenheim, Germany)	Disposable gloves, Peha/Soft®
Pfeiffer (Asslar, Germany)	Vacuum pump Duo5
Renner (Darmstadt, Germany)	Gel chamber
Sartorius (Göttingen, Germany)	Analytical balance A 200 S, microbalance MP3 and universal balance
Scientific Industries Inc. (New York, USA)	Vortexer Vortex Genie2
Tecan (Männedorf, CH)	Sunrise, NanoQuant (micro titer plate reader), Magellan6 (Software)
Thermo Fisher Scientific Inc. (Waltham, USA)	Autoclave, Varioklav®
Thermo Scientific (Langenselbold, Germany)	Sorvall Evolution RC Superspeed centrifuge
VWR (Darmstadt, Germany)	Power supply 300 V
VWR (Langenfeld, Germany)	Microtiter plates
Wilhelm Werner GmbH (Leverkusen, Germany)	Demineralizer, AQUADEM®

3.1.3 Bacterial strains and plasmids

Bacterial strains and plasmids used or constructed during this study are listed in Table 3 and Table 4

Table 3: Bacterial strains

Bacterial strain	Chromosomal genotype	Application	Reference
Chlamydophila pneumoniae GDI		Clinical strain for isolation of genomic DNA	AG Hegemann (University of Düsseldorf)
Escherichia coli W3110 (DSM3871)		Wild/type strain for preparation of peptidoglycan	DSMZ (Braunschweig)
Escherichia coli DH5α	fhuA2 _(argF-lacZ)U169, phoA, glnV44, Φ80 _(lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17	Cloning strain	Invitrogen (Carlsbad, USA)
Escherichia coli BL21 (DE3)	F-ompT gal dcm lonhsdSB(rB-mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Expression strain for cytoplasmic protein expression	Novagen/Merck (Darmstadt, Germany Eurolab)
Escherichia coli Top 10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-	Cloning strain	Invitrogen (Carlsbad, USA)
Escherichia coli JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB+ Δ(lac-proAB) e14- [F' traD36 proAB+ lacIq lacZΔM15] hsdR17(rK-mK+)	Cloning strain	New England Biolabs (Ipswich, USA)
Escherichia coli Lemo21 (DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS/ pLemo(CamR) λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5	Expression strain for toxic and transmembrane proteins	New England Biolabs (Ipswich, USA)

Bacterial strain	Chromosomal genotype	Application	Reference
	pLemo = pACYC184-PrhaBAD-lysY		
Escherichia coli BH101	F^-, cya-99, araD139, galE15, galK16, rpsL1 (Str^r), hsdR2, mcrA1, mcrB1	Reporter strain for the BACTH system (β-galactosidase assay)	Euromedex (Souffelweyersheim, France)
Escherichia coli DHM1	F-, cya-854, recA1, endA1, gyrA96 (Nal r), thi1, hsdR17, spoT1, rfbD1, glnV44(AS)	Reporter strain for the BACTH system (β-galactosidase assay)	Euromedex (Souffelweyersheim, France)
Escherichia coli XL-10 Gold	Tet^rΔ (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac the [F´ proAB $lacI^qZ$ΔM15 Tn10 (Tet^r) Amy Cam^r]	Cloning strain for the mutagenesis kit	Agilen Technologies (Waldbronn, Germany)
Micrococcus luteus DSM1790		For membrane preparation	DSMZ (Braunschweig, Germany)
Staphylococcus simulans 22 ATCC 12559		Indicator strain for the production of cell wall precursor	Kloos and Schleifer (1975) [122]

Table 4: Plasmids

Plasmid	Plasmid size [bp]	Resistance marker†	Description	Reference
pET-21b(+)	5442	Amp^R	Expression vector,T7 promoter, C-terminal His-tag	Novagen (Merck Eurolab)
pET-52b(+)	5227	Amp^R	Expression vector,T7 promoter, C-terminal His-tag, N-terminal *Strep*-tag	Novagen (Merck Eurolab)
pASK-IBA3	3247	Amp^R	Expression vector, *tet*A promoter, C-terminal *Strep*-tag II	IBA-BioTAGnology
pASK-IBA5	3260	Amp^R	Expression vector, *tet*A promoter, N-terminal *Strep*-tag II	IBA-BioTAGnology
pKT25	3442	Kan^R	Expression vector, *lac* promoter, derivative of plasmid pSU40, encodes T25 fragment upstream MCS‡	Euromedex, (Souffelweyersheim, France)
pKNT25	3469	Kan^R	Expression vector, *lac* promoter, derivative of plasmid pSU40, encodes T25 fragment downstream MCS‡	Euromedex, (Souffelweyersheim, France)
pUT18C	3017	Amp^R	Expression vector, *lac* promoter, derivative of plasmid pUC19, encodes T18 fragment upstream MCS‡	Euromedex (Souffelweyersheim, France)
pUT18	3023	Amp^R	Expression vector, *lac* promoter, derivative of plasmid pUC19, encodes T18 fragment downstream MCS‡	Euromedex (Souffelweyersheim, France)

Plasmid	Plasmid size [bp]	Resistance marker†	Description	Reference
pKT25 zip	3556	Kan^R	Derivative of pKT25, leucine zipper is fused to the T25 fragment	Euromedex (Souffelweyersheim, France)
pUT18C zip	3119	Amp^R	Derivative of pUT18C, Leucine zipper is fused to the T18 fragment	Euromedex (Souffelweyersheim, France)
pKJE7	7200	Cm^R	Chaperone plasmid	Takara Bio Inc. (Kyoto, Japan)
Recombinant plasmids				
pET21b-*pbp2* *PBP2* from *C. pneumoniae* GDI	8715	Amp^R	Expression vector, T7-promoter, PBP2 with C-terminal 6x His-Tag	This work
pET21b-*PBP3* *PBP3* from *C. pneumoniae* GDI	7404	Amp^R	Expression vector, T7 promoter, PBP3 with C-terminal 6x His-Tag	This work
pET21b-*dacF* *dacF* from *C. pneumoniae* GDI	6753	Amp^R	Expression vector, T7 promoter, PBP6a with C-terminal 6x His-Tag	This work
pET21b-*mreB* *mreB* from *C. pneumoniae* GDI	6543	Amp^R	Expression vector, T7 promoter, MreB with C-terminal 6x His-Tag	C. Otten
pASK-IBA3-*pbp2* *PBP2* from *C. pneumoniae* GDI	6520	Amp^R	Expression vector, *tet*A promoter, PBP2 with C-terminal *Strep*-Tag II	This work
pASK-IBA3-*PBP3* *PBP3* from *C. pneumoniae* GDI	5209	Amp^R	Expression vector, *tet*A promoter, PBP3 with C-terminal *Strep*-Tag II	This work
pASK-IBA3-*dacF* *dacF* from *C.*	4558	Amp^R	Expression vector, *tet*A promoter, PBP6a with C-	This work

Plasmid	Plasmid size [bp]	Resistance marker†	Description	Reference
pneumoniae GDI			terminal *Strep*-Tag II	
pASK-IBA3-*mreB* *mreB* from *C. pneumoniae* GDI	4348	Amp^R	Expression vector, *tet*A promoter, MreB with C-terminal *Strep*-Tag II	C. Otten
pASK-IBA5-*pbp2* *PBP2* from *C. pneumoniae* GDI	6533	Amp^R	Expression vector, *tet*A promoter, PBP2 with N-terminal *Strep*-Tag II	This work
pASK-IBA5-*PBP3* *PBP3* from *C. pneumoniae* GDI	5222	Amp^R	Expression vector, *tet*A promoter, PBP3 with N-terminal *Strep*-Tag II	This work
pASK-IBA5-*dacF* *dacF* from *C. pneumoniae* GDI	4571	Amp^R	Expression vector, *tet*A promoter, PBP6a with N-terminal *Strep*-Tag II	This work
pET-21b-*glyA* *glyA* from *C. pneumoniae* GDI	7002	Amp^R	Expression vector, T7 promoter, GlyA with C-terminal 6x His-Tag	This work
pET-52b-*glyA* *glyA* from *C. pneumoniae* GDI	6987	Amp^R	Expression vector, T7 promoter, GlyA with C-terminal 6x His-Tag and N-terminal *Strep*-tag	This work
Recombinant plasmids for the BACTH system				
pKNT25-*PBP3* *PBP3* from *C. pneumoniae* GDI	5431	Kan^R	Expression vector, *lac* promoter, PBP3 fused with T25-fragment at N-terminus	This work
pUT18-*PBP3* *PBP3* from *C. pneumoniae* GDI	5404	Amp^R	Expression vector, *lac* promoter, PBP3 fused *with* T18-fragment at N-terminus	This work

Plasmid	Plasmid size [bp]	Resistance marker†	Description	Reference
pKNT25-*dacF* *dacF* from *C. pneumoniae* GDI	4780	Kan^R	Expression vector, *lac* promoter, PBP6a fused *with* T25-fragment at N-terminus	This work
pUT18-*dacF* *dacF* from *C. pneumoniae* GDI	4753	Amp^R	Expression vector, *lac* promoter, PBP6a fused *with* T18-fragment at N-terminus	This work
pKNT25-*mreB* *mreB* from *C. pneumoniae* GDI	4570	Kan^R	Expression vector, *lac* promoter, MreB fused *with* T25-fragment at N-terminus	Dr. B. Henrichfreise
pUT18-*mreB* *mreB* from *C. pneumoniae* GDI	4543	Amp^R	Expression vector, *lac* promoter, MreB fused *with* T18-fragment at N-terminus	Dr. B. Henrichfreise
pKNT25-*mraY* *mraY* from *C. pneumoniae* GDI	4519	Kan^R	Expression vector, *lac* promoter, MraY fused *with* T25-fragment at N-terminus	Dr. B. Henrichfreise
pUT18-*mraY* *mraY* from *C. pneumoniae* GDI	4492	Amp^R	Expression vector, *lac* promoter, MraY fused *with* T18-fragment at N-terminus	Dr. B. Henrichfreise
pKNT25-*murG* *murG* from *C. pneumoniae* GDI	4543	Kan^R	Expression vector, *lac* promoter, MurG fused *with* T25-fragment at N-terminus	Dr. B. Henrichfreise
pUT18-*murG* *murG* from *C. pneumoniae* GDI	4516	Amp^R	Expression vector, *lac* promoter, MurG fused *with* T18-fragment at N-terminus	Dr. B. Henrichfreise

Plasmid	Plasmid size [bp]	Resistance marker†	Description	Reference
pKNT25-*amiA* *amiA* from *C. pneumoniae* GDI	4264	Kan^R	Expression vector, *lac* promoter, AmiA fused *with* T25-fragment at N-terminus	K. Schulz
pUT18-*amiA* *amiA* from *C. pneumoniae* GDI	4237	Amp^R	Expression vector, *lac* promoter, AmiA fused *with* T18-fragment at N-terminus	K. Schulz

† AmpR = Ampicillin resistance, KanR= Kanamycin resistance, CmR = Chloramphenicol resistance.

ǂ MCS = multiple cloning site

3.1.4 Culture Media

Table 5 includes the growth media used for the cultivation of the bacterial strains deployed in this work. Unless otherwise indicated, the culture media were dissolved in deionized distilled water sterilized by autoclaving for 20 min. at 121°C. Heat-sensitive substances, sugars and antibiotics were dissolved separately in appropriate solvents and added to the autoclaved media after they cooled down to 50°C

Table 5: Culture media

Culture medium	Composition
Luria-Bertani (LB)-agar	10 g/l NaCl, 10 g/l trypton, 5 g/l yeast extract, 15 g/l agar-agar, pH 7.5
Luria-Bertani (LB)-medium	10 g/l NaCl, 10 g/l trypton, 5 g/l yeast extract, pH 7.5
NZY^+ broth	10 g/l NZ amine (casein hydrolysate), 5 g/l yeast extract, 5 g/l NaCl, 12,5 ml $MgCl_2$ (1 M), 12.5 ml $MgSO_4$ (1 M), 20 ml Glucose (20% (w/v)), pH 7.5
SOC-medium	20 g/l trypton, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose, pH 7.5
MacConkey (Difco Laboratories)/maltose medium	40 g/l MacConkey base, 10g/l maltose, 0.5 mM IPTG

3.1.5 Antibiotics

Antibiotics used in this study for the preparation of selective media are listed in Table 6.

Table 6: Antibiotics

Antibiotic	Stock solution (mg/ml)	Solvent	Supplier/Source
Ampicillin	100	Distilled water	Sigma-Aldrich
Kanamycin	50	Distilled water	Sigma-Aldrich
Chloramphenicol	100	Ethanol (100 %)	Sigma-Aldrich h
Anhydrotetracycline	2	Ethanol (100 %)	IBA-BioTAGnology

3.2 MICROBIOLOGICAL METHODS

3.2.1 Cultivation bacterial strains

All bacterial strains used in this study were stored as glycerol cultures (40 % v/v) at -70°C. Streaking on LB agar plates was used to cultivate *E. coli* strains from glycerol cultures. Inoculated plates were incubated at 37°C overnight and then kept at 4°C. Selective pressure of the appropriate antibiotics (Table 6) was used to propagate *E. coli* strains that harbor resistance plasmids (Table 4).

Unless otherwise indicated, cultivation in liquid media was performed aerobically at 37°C with shaking at 150 rpm in a shaking water bath (CEROMAT® H). To prepare a main culture for protein over-expression, 2% (v/v) inoculum from an overnight preculture was used to prepare the main culture.

3.2.2 Preparation of glycerol cultures

For long-term storage of bacterial strains, separate colonies of the corresponding strains from fresh LB agar plates were mixed with pre-autoclaved LB medium and 85% glycerol (1:1) in screw-capped freezing vials. Alternatively, 0.6 ml logarithmic-phase *E. coli*

cultures in LB medium were pipetted into pre-autoclaved screw-capped freezing vials containing 0.4 ml 100% glycerol and vortexed vigorously. Glycerol cultures were stored directly after preparation at -70°C.

3.2.3 Sterilization

The sterilization of glassware was carried out at 200°C for 4 hours in hot air oven. Culture media, buffers, pipette tips and centrifugation bottles were autoclaved at 121°C for 20 minutes. Stock solutions containing heat-sensitive substances like antibiotics and sugars were sterilized by filtration through 0.2 μm membrane filters.

3.2.4 Measurement of optical density of liquid cultures

The cell density of bacterial suspensions was determined by measuring the optical density (OD) of appropriately diluted samples using a spectrophotometer at a wavelength of 600 nm [OD_{600}], against the respective suspending medium as a blank.

3.3 MOLECULAR BIOLOGICAL METHODS

3.3.1 Isolation of genomic DNA

Genomic DNA of *C. pneumoniae* GDI was kindly supplied by Prof. Dr. Hegemann (University of Düsseldorf, Germany).

3.3.2 Isolation of plasmid DNA from *E. coli* cultures

Plasmid DNA was isolated from *E. coli* cultures using the Qiaprep® Miniprep kit (Qiagen GmbH), according to the manufacturer's instructions. The Qiaprep miniprep procedure uses a modified alkaline lysis method of Birnboim and Doly [123]. In this method, the bacterial cells, after harvesting and resuspension, are lysed in NaOH/SDS buffer. The anionic detergent SDS denatures phospholipids and protein contents of the cell membrane leading to cell lysis and release of cell content. The alkaline pH does not help only to break down the cell wall but also to denature both genomic and plasmid DNA inside the cells. Acidified potassium acetate buffer is then used to neutralize the lysate and to adjust it to high salt content. Under these conditions, small circular plasmid DNA renatures while SDS denatured proteins, chromosomal DNA, and cellular debris are precipitated and cleared by centrifugation. DNA in the cleared lysate can bind selectively to silica membrane in the Qiaprep columns. After washing steps, DNA was eluted in 50 µl distilled water (50°C). The plasmid DNA was used directly for restriction reactions, transformation, sequencing or stored at -20°C.

3.3.3 Photometric determination of DNA concentration

DNA concentration was measured through photometric analysis by Nanophotometer (Implen). Because of its nitrogenous bases content, DNA absorbs in the Ultraviolet (UV) range, at a wavelength of 260 nm. A reading of 1.0 at 260 nm corresponds to 50 µg/ml of pure double stranded DNA and the more concentrated the DNA solution, the more UV light it absorbs [124].

DNA purity can be estimated by calculating the ratio A260/A280. A ratio of 1.8 represents a high-purity DNA. Ratios lower than 1.8 indicate contamination with proteins while ratios higher than 1.8 indicate contamination with RNA.

3.3.4 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis is a method to separate nucleic acid fragments by length. In this method, DNA fragments are separated by applying an electric field to move negatively charged molecules from the cathode to the anode through an agarose matrix [124]. Several factors affect the rate of migration of DNA through agarose gels, including:

- the size of the DNA. Smaller DNA molecules migrate faster than larger ones because they can move easily through gel pores,
- the concentration of agarose. The higher the agarose concentration, the slower the rate of DNA migration,
- the conformation of the DNA. Superhelical circular, nicked circular and linear DNA conformations migrate in agarose gel in different rates,
- applied voltage and the type of agarose.

DNA samples analyzed by gel electrophoresis should be mixed with a loading buffer, which helps DNA to sink in the sample well and visualizes the migration during electrophoresis through two tracking dyes. Ethidium bromide is a fluorescent dye that intercalate non-specifically between DNA bases causing DNA to fluoresce under UV light (254 nm).

In this study, 1% agarose (Fermentas GmbH) was cooked in 1X tris acetate buffer (TAE) using microwave (AEG) until agarose melted completely. After cooling down to 60°C, ethidium bromide was added to a final concentration of 0.5 μg/ml. The gel was poured into a casting tray containing a suitable comb and allowed to solidify at room temperature. Before loading on the gel, samples were mixed with loading buffer. Electrophoresis was done in an electrophoresis chamber filled with 1X TAE buffer. With the help of DNA ladder (Table 7), the size of the analyzed DNA fragments was determined.

Table 7: DNA ladder for agarose gel electrophoresis

DNA ladder	DNA-Fragments (bp)	Source
GeneRuler™ 1kb DNA Ladder	250, 500, 750, 1.000, 1.500, 2.000, 2.500, 3.000, 3.500, 4.000, 5.000, 6.000, 8.000, 10.000	Fermentas GmbH

3.3.5 Polymerase chain reaction (PCR)

The polymerase chain reaction is a technique developed by Kary Mullis [125] to amplify a specific DNA segment *in vitro*. In addition to the target DNA, a PCR reaction requires several other ingredients. A pair of primers that hybridize the target DNA at areas flanking the segment to be amplified, free deoxyribonucleotides (dNTPs), and heat stable DNA polymerase which uses the dNTPs to fill the gap between both primers. The PCR reaction relies on repeated (30-40 cycles) thermal cycling. Each cycle consists of three thermal steps:

- DNA denaturation, samples are heated up more than 95°C to separate the DNA double strands,
- annealing step, in which the reaction is cooled down to 50-70°C to allow the primer pair to hybridize their DNA target,
- DNA extension, in which the reaction temperature is raised up to 72°C, at which DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs in 5' to 3' direction.

The product of each cycle can be used as a template for the next cycles allowing almost exponential amplification. The thermal cycles are usually preceded by a single step of initial denaturation and followed by a step of final extension.

In this study, Phusion High-Fidelity PCR kit (NEB) was used to prepare the PCR reactions according to the manufacturer recommendations. The Phusion DNA polymerase generates long templates with high accuracy and speed. The PCR reactions were achieved using iCycler (BioRad).

DNA primers used to amplify DNA segments during this study were synthesized by Eurofins MWG Operon and listed with their specific restriction enzymes, annealing temperatures and extension time in Table 8.

The PCR reaction components and pipetting scheme are shown in Table 9. The exact thermal profile used for each pair of primers is shown in Table 10. During the design of primers to amplify DNA for cloning purpose, different recognition site was introduced in each of the primer pair to direct the DNA fragment during cloning.

3.3.5.1 Colony PCR

Colony PCR was used to detect positive clones during cloning process. Positive clones are bacterial clones that received a vector in which an insert was introduced during ligation step. Colony PCR uses the same reaction as normal PCR with minor modifications. A small amount of the bacterial colony is used as template instead of the genomic DNA in the PCR reaction (Table 9) and the initial denaturation step is extended to 5 min instead of 30 seconds (Table 10) to help the release of the bacterial DNA before the amplification cycles.

Table 8: Oligonucleotide primers

Gene (Size bp) Vector	Oligonucleotide	Sequence (5'-3')§	Restriction enzyme	Annealing temp (°C)	Extension time (sec)
PBP2 (3273)	PBP2-Cp-IBA3F	ATGGTAGGTCTCAAATGAAACG	BsaI†	67.5	90
pASK-IBA3	PBP2-Cp-IBA3R	ACCGAAAAAATTTCCTATTTAT ATGGTAGGTCTCAGCGCTTCCCC TTAAAAAAGATTCTCTTTGTT	BsaI		
PBP2 (3273)	PBP2-Cp-IBA5F	ATGGTAGGTCTCAGCGCCATGA AACGACCGAAAAAATTTCCTAT	BsaI	67.5	90

Gene (Size bp) Vector	Oligonucleotide	Sequence (5'-3')§	Restriction enzyme	Annealing temp (°C)	Extension time (sec)
pASK-IBA5	PBP2-Cp-IBA5R	ATGGTAGGTCTCATATCATCCCCTTAAAAAAGATTCTCTTTGTT	BsaI		
PBP2 (3273)	PBP2-Cp-pET-F	CATAATATCTATCATATGAAACGACCG	NdeI	59	90
pET-21b	PBP2-Cp-pET-R	AAAGCGGCCGCTCCCCTTAAAAAAG	NotI		
PBP3 (1962)	PBP3-Cp-F	TATCATATGAGCTACCGTAAACG	NdeI	57	60
pASK-IBA3	PBP3-Cp-IBA3R	GTTGAATTCATGAGCTACCGTAAACGTTC	EcoRI		
PBP3 (1962)	PBP3-Cp-F	TATCATATGAGCTACCGTAAACG	NdeI	57	60
pASK-IBA5	PBP3-Cp-IB5R	GTTGAATTCGATGAGCTACCGTAAACGTT	EcoRI		
PBP3 (1962)	PBP3-Cp-F	TATCATATGAGCTACCGTAAACG	NdeI	57	60
pET-21b	PBP3-Cp-R	AGACTCGAGCCTCGTTCCCCCTTG	XhoI		
dacF (1311)	PBP6a-IBA3-F	ATGGTAGGTCTCAAATGAAAAGACCTTTTTTTTACCTATCTATGC	BsaI	65	40

Gene (Size bp) Vector	Oligonucleotide	Sequence (5'-3')§	Restriction enzyme	Annealing temp (°C)	Extension time (sec)
pASK-IBA3	PBP6a-IBA3-R	ATGGTAGGTCTCAGCGCTGATTTTAGAATAGTGTTTTAAATTTTTATA	BsaI		
dacF (1962)	PBP6a-IBA5-F	ATGGTAGGTCTCAGCGCCATGAAAAGACCTTTTTTTACCTATCTA	BsaI		
pASK-IBA5	PBP6a-IBA5-R	ATGGTAGGTCTCATATCAGATTTTAGAATAGTGTTTTAAATTTTTATA	BsaI	65	40
dacF (1962)	PBP6a-pET-F	CAAAAGTCCGGCTAGCAAAAGACCTTTT	NheI		
pET-21b	PBP6a-pET-R	GAAACTCGAGGATTTTAGAATAG	XhoI	55	40
murF (1344)	MurF-Cp-F	AAACCGCGGGGCGAGCTATGTTGCTTG	SacII		
pASK-IBA5	MurF-Cp-R	CTAAGCCATGGAAAACAAGCTAATAAG	NcoI	55	40
murF (1344)	MurF-Cp-F	GCAAAATGCTAGCCGAGCTATGTTGC	NheI		
pET-21b	MurF-Cp-R	CCTAGCGGCCGCAAAACAAGCTAATAAG	NotI	65	40

Gene (Size bp) Vector	Oligonucleotide	Sequence (5'-3')§	Restriction enzyme	Annealing temp (°C)	Extension time (sec)
glyA (1560) pET-21b	GlyA-pET21-F	CGTCTTTAGAAGCATATGCTAAAAG	NdeI	58	40
	GlyA-pET21-R	GTCTCTGCGGCCGCAACTAAAGCTTC	NotI		
glyA (1560) pET-52b	GlyA-pET52-F	CTTTAGAAGCCCGGGCTAAAAGTTTTTG	SmaI†	61	60
	GlyA-pET52-R	GTCTCTCCGAGCTCAACTAAAGCTTC	SacI		
PBP3 (1962) BACTH	PBP3-BACTH-F	GTCTTTTACTGCAGTAGCTACCGTAAACG	PstI	65	60
	PBP3-BACTH-R	ATAGAGGATCCTCCCTCGTTCCCCCTTG	BamHI		
dacF (1311) BACTH	PBP6A-BACTH-F	CAAAAGTCCTCTAGAGAAAAGACCTTTT	XbaI	57.5	40
	PBP6A-BACTH-R	AAAAGAGGTACCAAGATTTTAGAATAG	KpnI		

§ The underlined sequence shows the recognition site of the used restriction endonucleases.

† All restriction endonucleases used cut within their recognition site to produce sticky ends with the exception of BsaI, which cuts outside its recognition site producing sticky ends and SmaI, which cuts inside its recognition site producing blunt ends.

Table 9: Pipetting scheme for PCR reactions

Component	PCR	Colony PCR
H_2O	Add to 50 µl	Add to 15 µl
5x Phusion HF Buffer	10 µl	3 µl
10 mM dNTPs	1 µl	0.3 µl
Forward primer (10 pmol/ µl)	2.25 µl	0.75 µl
Reverse primer (10 pmol./ µl)	2.25 µl	0.75 µl
Template DNA	50 ng	Small amount of the colony
Phusion DNA polymerase	1 unit	0.3 unit

Table 10: PCR thermal profile

PCR step		Temperature (°C)	Time	Repetitions
Initial denaturation		98	30 sec	1
PCR amplification cycles	Denaturation	98	10 sec	35 cycles
	Annealing	Table 8	20 sec	
	Extension	72	Table 8	
Final extenion		72	10 min	1
Cooling down		4	∞	1

3.3.5.2 Purification of DNA fragments

DNA fragments from PCR reactions, agarose gel or enzymatic reactions were purified using MinElute PCR purification kit (Qiagen) according to the manufacturer instructions. During DNA purification, all enzymes, salts and oligomers smaller than 70 bp were removed during the column loading and washing. DNA fragments with sizes between 70 bp and 4 kb were eluted in 10-15 µl of distilled water.

3.3.6 Site directed mutagenesis

Cysteine 345 in chlamydial MreB was changed to alanine, producing C345A MreB mutant, using QuikChange® Lightning Site-Directed Mutagenesis kit (Agilent Technologies) according to manufacturer's instructions. The basic procedure (Figure 12) utilizes a double-stranded DNA plasmid with insert of interest and two synthetic oligonucleotide

primers containing the desired mutation. The primers, each complementary to opposite strands of the vector, were extended with thermal cycling to produce a mutated plasmid containing staggered nicks. The product of the thermal cycling step was treated with DpnI to digest the parental DNA. The nicked vector DNA containing the desired mutations was then transformed into XL-Gold ultracompetent cells.

The mutagenic primers used to produce C345A MreB mutant are shown in Table 11.

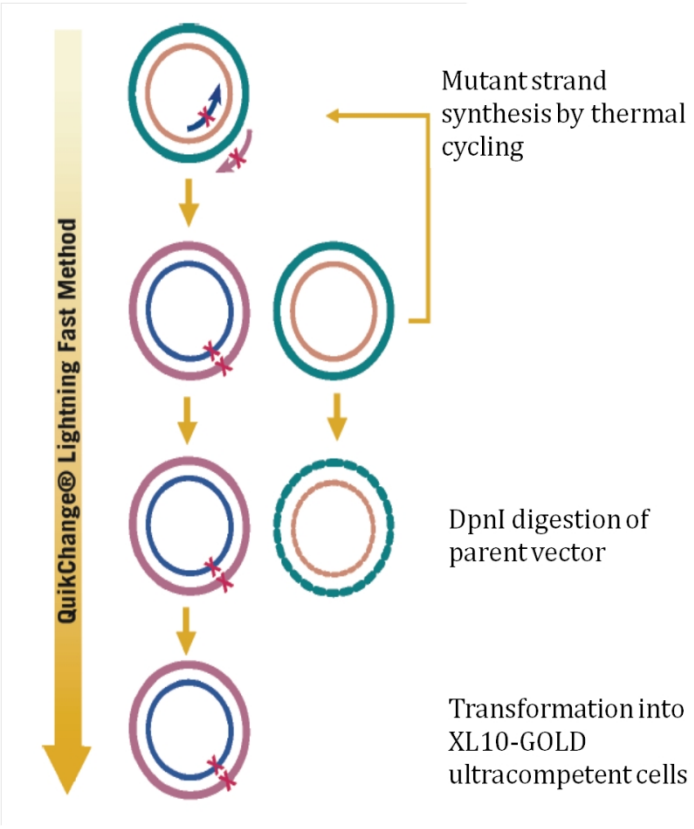

Figure 12: Schematic presentation of mutagensis kit principle

Table 11: Mutagenic primers used for C345A *mreB* synthesis

Oligonucleolide	**Sequence (5'-3')§**
mreB C345A sense	CATCCTTTGCTGGCAGTTGCTTTAGGAACCGGGAAAGC
mreB C345A antisense	GCTTTCCCGGTTCCTAAAGCAACTGCCAGCAAAGGATG

§ The underlined sequence represents the position of mutation

3.3.7 Cloning

3.3.7.1 DNA restriction with restriction endonucleases

Restriction endonucleases are enzymes that recognize specific nucleotide sequences known as recognition sites and produce a double-stranded cut in the DNA by hydrolyzing the DNA-phosphodiester bonds. In bacteria, it is considered as a defense mechanism against foreign DNA like invading viruses [126]. The recognition site in most cases is palindormic and consists of 4-8 nucleotides. According to their properties, DNA restriction endonucleases are classified into three main types. While type I and III cleave DNA outside their recognition site, type II enzymes cleave DNA within or at short specific distance from their recognition sites.

All restriction enzymes used in this work are of type II (Table 8) and have recognition site of 6 palindormic nucleotides except NotI (8 nucleotides). All cut DNA within the recognition site with the exception of BsaI (cuts outside the recognition site) to produce sticky-ended DNA with the exception of SmaI (produce blunt-ended DNA). All restriction reactions were performed according to the manufacturer's instructions.

3.3.7.2 Dephosophorylation of the linearized vectors

During ligation step, the ligase enzyme catalyzes the formation of a phosphodiester bond between 5' phosphate group and 3' hydroxyl group of two adjacent DNA strands. Thus, the removal of 5' phosphate from a linear plasmid can minimize self-ligation of plasmid.

In this work, antarcatic phosphatase (NEB) was used to dephosphorylate restricted vectors following the manufacturer's instructions.

3.3.7.3 Purification of DNA after enzymatic reactions

At the end of DNA restriction and dephophorylation reactions, DNA was purified using MinElute Reaction Cleanup columns (Qiagen) according to the manufacturer's instructions. DNA fragments were purified from enzymes and salts as illustrated in 3.3.5.2.

3.3.7.4 DNA ligation

T4 DNA ligase is an enzyme that links DNA fragments together by forming a phosphodiester covalent bond between 5' phosphate and 3' hydroxyl groups of two adjacent fragments [124]. The enzyme can catalyze the ligation of both complementary sticky-ended DNA fragments, produced by cutting both vector and insert using the same restriction enzyme, and blunt-ended DNA.

In this work, T4 DNA ligase (Roche) was used according to the manufacturer's instructions.

3.3.7.5 Concentrating DNA samples

DNA samples with low concentration were concentrated using SpeedVac (Savant) at 35°C for 30-45 minutes under vacuum.

3.3.8 Expression vectors

Several expression vectors were used during this work (Table 4)

3.3.8.1 pASK-IBA3 and pASK-IBA5

pASK-IBA vectros (IBA-BioTAGnology) are *Strep*-tag II expression vectors that carry the promoter/operator region from the *tetA* resistance gene. These vectors provide tight repression of the promoter by the constitutive expression of the *tet* repressor gene. The over-production of the heterologous protein can be fully induced by adding anhydrotetracycline at a concentration that is not antibiotically effective (200 ng/ml). The strength of the *tetA* promoter is nearly 25% the activity of the T7 promoter.

pASK-IBA3 and pASK-IBA5 (Figure 13) introduce a C-terminal and N-terminal *Strep*-tag II to the cloned protein respectively. The *Strep*-tag II is a short peptide (8 amino acids, WSHPQFEK), which binds with high selectivity to *Strep*-Tactin, an engineered streptavidin. Thus, the binding affinity to *Strep*-Tactin can be used for purification of recombinant proteins. Both vectors encode ampicillin resistance gene and use ampicillin as a selection marker.

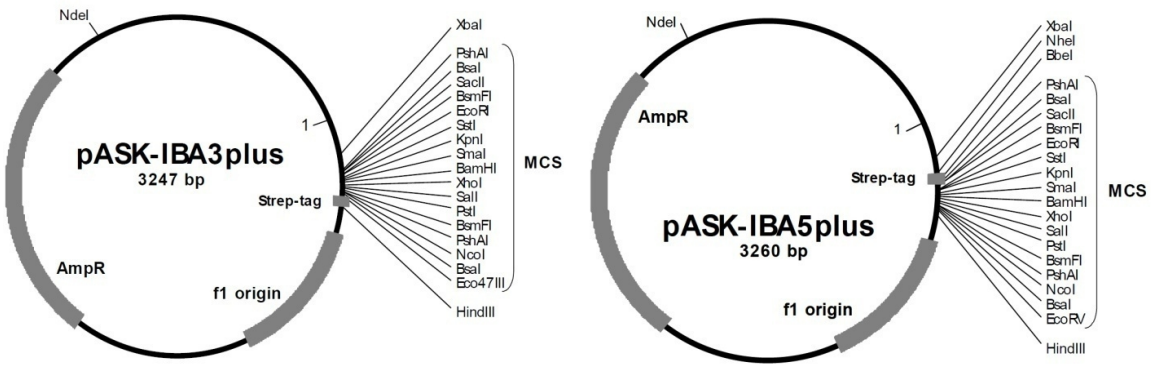

Figure 13: Vector map of pASK-IBA3 plus and pASK-IBA5 plus* expression vectors (IBA-BioTAGnology)

* The plus vectors are identical to the non-plus vectors but contain an improved translation initiation site.

3.3.8.2 pET-21b and pET-52b expression vectors

pET vectors (Novagen) are vectors in which target genes are cloned under control of the strong bacteriophage T7 promoter which is specific to T7 RNA polymerase provided by the host *E. coli* strains. The expression in pET system is under the control of *lac* operator and promoter. The addition of isopropyl-β-thiogalactopyranoside (IPTG) induces the expression of the chromosomally encoded T7 polymerase, which replaces the repressor from the vector *lac* operon starting protein transcription and translation.

pET-21b vector (Figure 14) introduces a hexa histidine-tag (His-tag) at the C-terminus of the recombinant protein which facilitates the protein purification using nickel nitroactic acid (Ni-NTA) resin or TALON metal affinity resin. On the other hand, pET-52b vector (Figure 14) encodes two fusion proteins; a C-terminal His-tag and an N-terminal *Strep*-tag; allowing double protein purification using Ni-NTA resin and *Strep*-Tactin sepharose.

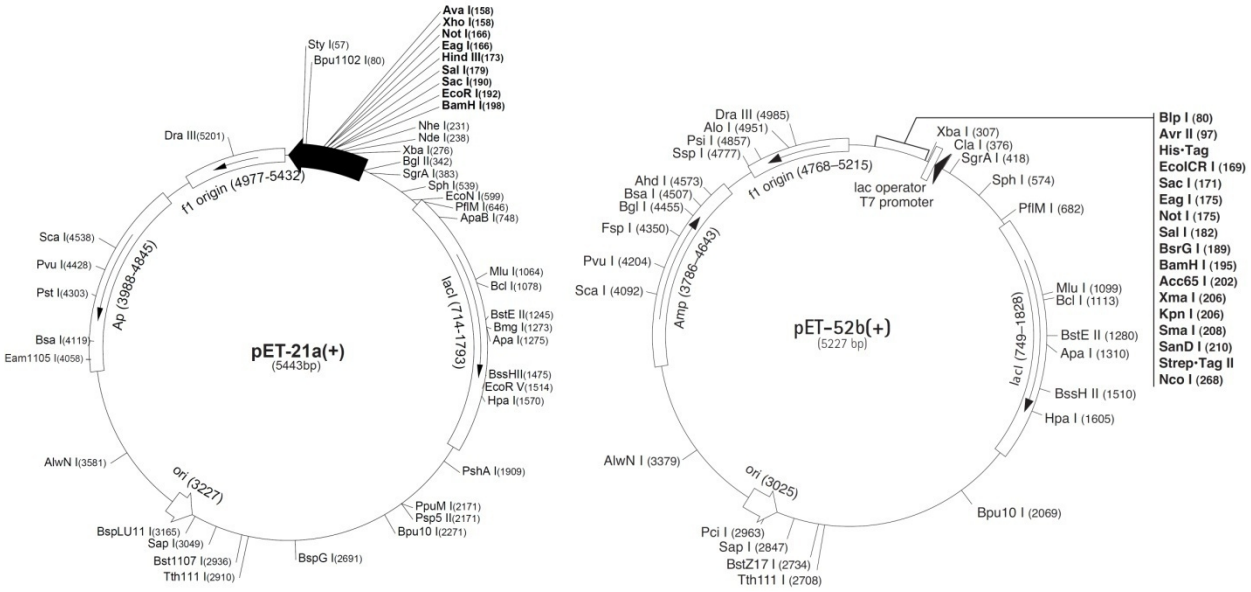

Figure 14: Vector map of pET-21a* and pET-52b expression vectors (Navagen)

* pET-21b is similar to pET-21a but one nucleotide less in size (5442 bp).

3.3.8.3 Sequencing

The confirmation of the correctness of the cloned genes was achieved by sequencing the plasmid-DNA at SeqLab (Göttingen). Primers used for sequencing are listed in Table 12.

Table 12: Primers for sequencing

Vector	Primer name	Sequence (5'-3')
pASK-IBA3 and pASK-IBA5	5IBA3	GAGTTATTTTACCACTCCCT
	IBA2r	CGCAGTAGCGGTAAACG
pET-21b and pET-52b	T7	TAATACGACTCACTATAGGG
	T7 term	GCTAGTTATTGCTCAGCGG
BACTH-pUT18	pUT18_F	TTTATGCTTCCGGCTCGTATGTT
	pUT18_R	CAAGTCGATGCGTTCGCGAT
BACTH-pKNT25	pKNT25_F	CTTTACACTTTATGCTTCCG
	pKNT25_R	CTTCGCCACGGCCTTGATGC

3.3.9 Transformation in competent bacteria

During this work, transformation of plasmid DNA into *E. coli* strains, during cloning or for protein over-expression, was achieved using chemo-competent *E. coli* cells.

3.3.9.1 Preparation of chemo-competent *E. coli*

LB medium (200 ml) was infected with 1% (v/v) overnight culture of *E. coli* strains. Culture was incubated in a shaking water bath (GFL) at 37°C and about 100 rpm until OD_{600} of 0.4-0.5. The cells were harvested by centrifugation (Heraeus Biofuge 28RS, rotor: 3751, 5300 rpm, 20 minute, 4°C). Cell pellet was resuspended in 80 ml ice-cold 0.1 M $CaCl_2$ solution. Cell suspension was incubated on ice for 20 minutes and then harvested by centrifugation (see above). Pellet was resuspended in 2 ml 0.1 M $CaCl_2$ solution in 15% glycerol and incubated for an hour on ice. Chemo-competent cells were divided as 60 µl aliquots in pre-cooled eppendorfs and stored directly at -70°C.

3.3.9.2 Transformation in $CaCl_2$-competent *E. coli*

An aliquot of $CaCl_2$-competent cells was thawed gently on ice. Plasmid DNA (10 ng or 5 µl ligation product) was added to the cells and incubated on ice for 30 minutes followed by heat shock in water bath (Julabo) at 42°C for 90 seconds. Cells were incubated on ice for 2 minutes. SOC medium (1 ml) was added to the cells and the transformation tube was incubated in a shaking incubator (Braun) for an hour at 37°C and 140 rpm. At the end, appropriate volumes (2-250 µl) of the transformation reaction were plated on LB agar supplemented with the proper concentration of the corresponding resistance marker carried by the transformed plasmids. LB agar plates were incubated at 37°C overnight.

3.3.10 Over-production and purification of *Strep*-tag fusion proteins from pASK-IBA vectors

During this work, several chlamydial genes were cloned in pASK-IBA vectors, which add a *Strep*-tag to the cloned protein. Based on the binding selectivity of *Strep*-tag to *Strep*-Tactin, *Strep*-Tactin sepharose (IBA-BioTAGnology) was used to purify recombinant proteins under physiological conditions. After application of cleared cell lysate on *Strep*-Tactin column and washing steps, the recombinant protein was eluted with low concentration of desthiobiotin (2.5 mM).

3.3.10.1 Over-production and purification of cytoplasmic fusion proteins *Strep*-tagged MreB and *Strep*-tagged MurF from *C. pneumoniae*

The expression strain *E. coli* BL21 (DE3) was transformed with pASK-IBA3-*mreB* or pASK-IBA5-*murF* plasmids. A preculture was prepared in LB medium supplemented with 100 mg/l ampicillin and incubated in shaking water bath (GFL) at 30°C overnight. Expression culture was prepared using 2% (v/v) inoculum from the preculture in fresh LB medium supplemented with 100 mg/l ampicillin. The expression culture was incubated in shaking water bath at 30°C until OD_{600} of 0.5-0.6. Anhydrotetracycline (200 ng/ml) was added to induce the recombinant protein production. Culture was incubated in shaking water bath at 20°C for 20 hours. Cells were harvested by centrifugation (Sorvall Evolution Superspeed, rotor: SLC-6000, 7000 rpm, 15 min, 4°C). Cell pellets were stored at -20°C until used for preparation of cleared lysate.

All buffers used for the purification of the *Strep*-tagged proteins are listed with their composition in Table 13. Cleared lysate was prepared by allowing frozen cells to thaw on ice for 15 minutes. Cell pellet from 1 liter culture was resuspended in 10 ml buffer W. Cell suspension was sonicated for 10 cycles of 20 seconds separated by 1 minute break on ice using Ultrasonic homogenizer UP50H (Branson). DNase I (20 mg/l) and RNase A (20 mg/l) were added and suspension was incubated on ice for 15 minutes, .then centrifuged (Heraeus, rotor: 3746, 13500 rpm, 30 minutes, 4°C). The clear supernatant, which contains the soluble proteins, was separated from the insoluble cell components.

All the following steps for protein purification were performed at 4°C. The cleared lysate was added to a pre-equilibrated *Strep*-Tactin column, where the fusion protein containing *Strep*-tag could bind to the *Strep*-Tactin sepharose. The column was washed 5 times with 1 column bed volume (CV) of buffer W to remove non-specifically bound proteins to the *Strep*-Tactin. Finally, the *Strep*-tagged protein was eluted with 3 CV of buffer E and collected in 0.5 CV fractions. The *Strep*-Tactin column was regenerated using 10 CV of buffer R.

At the end of purification steps, *Strep*-tagged MreB elution fraction were stored on ice at 4°C, while *Strep*-tagged MurF was mixed with 98% glycerol to final glycerol concentration of 20% (v/v) and stored at -20°C.

Table 13: Buffers used for purification of *Strep*-tagged proteins

Buffer	Composition
Buffer W (lysis and wash buffer)	100 mM Tris/HCl pH8.0, 150 mM NaCl, 1 mM EDTA
Buffer E (elution buffer)	100 mM Tris/HCl pH8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin
Buffer R (regeneration buffer)	100 mM Tris/HCl pH8.0, 150 mM NaCl, 1 mM EDTA, 1 mM hydroxy-azophenyl-benzoic acid (HABA)

3.3.10.2 Over-production and purification of *Strep*-tagged PBPs from *C. pneumoniae*

All the steps for the over-production of *Strep*-tagged PBPs from *C. pneumoniae* were the same as described in section 3.3.10.1 with some modifications. Several incubation temperatures for the overnight culture and the expression culture were tried (20°C, 25°C, 30°C and 37°C). Induction was performed over different periods of incubations (3 hours, 5 hours and 20 hours). Induction was started at different OD_{600} (0.4, 0.5, and 0.6).

Some modifications were introduced to the steps for the preparation of the cleared lysate described in section 3.3.10.1. Having a predicted membrane anchor, PBPs were expected to need detergents to be dissolved. Buffer W containing different concentrations of one or two detergents (triton x100, sodium lauroylsarcosine, DDM, CHAPS, and tween 20) were tried to prepare the cleared lysate.

3.3.11 Over-production and purification of His-tag fusion protein from pET-21b and pET-52b vectors

During this work, chlamydial genes encoding MreB, PBPs, MurF, and GlyA were cloned in pET-21b and/or pET52b vector(s), which introduce(s) a His-tag at the C-terminal of the cloned protein. The His-tag facilitates the protein purification by binding to immobilized metal chelate affinity chromatography based on Ni^{2+} or Co^{2+} ions such as Ni-NTA or TALON resins respectively. After washing steps with buffer containing low

concentrations of imidazole, His-tagged proteins are eluted with high concentrations of imidazole, which competitively replaces the bound His-tag.

3.3.11.1 Over-production and purification of MreB-His$_6$ and C345A MreB-His$_6$ mutant from *C. pneumoniae*

The plasmid pET-21b-*mreB* was transformed into *E. coli* BL21 (DE3). A preculture was prepared in LB medium supplemented with 100 mg/l ampicillin. The culture was incubated in a shaking water bath (GFL) at 30°C overnight. The expression culture was prepared using 2% (v/v) inoculum of the preculture in a fresh LB medium supplemented with 100 mg/l ampicillin. The expression culture was incubated in shaking water bath at 30°C until OD_{600} of 0.5-0.6. IPTG (0.5 mM) was added to induce the recombinant protein production. Culture was incubated in shaking water bath at 20°C for 4-5 hours. Cells were harvested by centrifugation (Sorvall Evolution Superspeed, rotor: SLC-6000, 7000 rpm, 15 min, 4°C). Cell pellet were stored at -20°C until used for preparation of cleared lysate.

All buffers used for the purification of the His-tagged proteins are listed with their composition in Table 14. Cleared lysate was prepared by allowing frozen cells to thaw on ice for 15 minutes. Cell pellet of 1 liter culture was resuspended in 10 ml lysis buffer, and lysozyme was added to a final concentration of 0.5 mg/ml. Cells were allowed to lyse at room temperature for 30 minutes. Cell suspension was sonicated for 6 cycles of 15 seconds separated by 1 minute break on ice using Ultrasonic homogenizer UP50H (Branson). DNase I (20 mg/l) and RNase A (20 mg/l) were added and suspension was incubated on ice for 15 minutes. The suspension was then centrifuged (Heraeus, rotor: 3746, 13500 rpm, 30 minutes, 4°C). The clear supernatant, which contains the soluble proteins, was separated from the insoluble cell components.

All the following steps for protein purification were performed at 4°C. The cleared lysate from 1 liter culture was mixed with 1 ml pre-equilibrated Ni-NTA resin and incubated with mild shaking at 4°C for 1 hour. The Ni-NTA resin was packed in gravity flow column, washed with 5 ml of wash buffer 1 followed by 5 ml of wash buffer 2. His-tagged protein was eluted using 3 ml of elution buffer and collected in 0.5 ml fractions.

At the end of purification steps, His-tagged protein elution fractions were stored on ice at 4°C.

Table 14: Buffers used for the purification MreB-His$_6$

Buffer	Composition
Lysis buffer	50 mM Tris/HCl pH 8, 300 mM NaCl
Wash buffer 1	50 mM Tris/HCl pH 8, 300 mM NaCl, 10 mM imidazole
Wash buffer 2	50 mM Tris/HCl pH 8, 300 mM NaCl, 20 mM imidazole
Elution buffer	50 mM Tris/HCl pH 8, 300 mM NaCl, 250 mM imidazole

3.3.11.2 Over-production and purification of MurF-His$_6$ from *C. pneumoniae*

All steps for the over-production of His-tagged MurF from *C. pneumoniae* are the same as described in section 3.3.11.1.

Due to the formation of inclusion bodies, the His-tagged protein was purified from the pellet remaining after preparation of the cleared lysate as described in section 3.3.11.1. The pellet was washed with 10 ml lysis buffer, then resuspended in 10 ml lysis buffer containing 0.5% (w/v) sodium lauroylsarcosine, incubated on ice for 15 minutes with vortexing in between, followed by centrifugation (Heraeus, rotor: 3746, 13500 rpm, 30 minutes, 4°C). The clear supernatant, which contains the His-tagged protein, was separated from the insoluble components.

The protein purification steps are the same as described in section 3.3.11.1 using buffers listed in Table 15.

After purification, MurF was used directly for co-pelleting assay with MreB or mixed with 98% glycerol until a final concentration of 20% (v/v) and stored at -20°C.

Table 15: Buffers with sodium lauroylsarcosine used for His-tagged protein purification

Buffer	Composition
Lysis buffer	50 mM sodium phosphate buffer pH 7.5, 300 mM NaCl
Wash buffer 1	50 mM sodium phosphate buffer pH 7.5, 10 mM imidazole, 0.5% (w/v) sodium lauroylsarcosine

Buffer	Composition
Wash buffer 2	50 mM sodium phosphate buffer pH 7.5, 20 mM imidazole, 0.5% (w/v) sodium lauroylsarcosine
Elution buffer	50 mM sodium phosphate buffer pH 7.5, 250 mM imidazole, 0.3% (w/v) sodium lauroylsarcosine

3.3.11.3 Over-production and purification of His-tagged PBPs from *C. pneumonaie*

The plasmid pET-21b-*PBP2*, -*PBP3*, or -*dacF* was co-transformed with chaperone plasmid pKJE7 (TaKaRa) into *E. coli* BL21 (DE3). pKJE7 plasmid (Figure 15) encodes three chaperone proteins DnaK, DnaJ and GrpE. Chaperones have been demonstrated to be involved in protein folding process and preventing protein aggregation. Co-expression of a target protein with chaperones was shown to increase the recovery of expressed proteins in the soluble fraction[127].

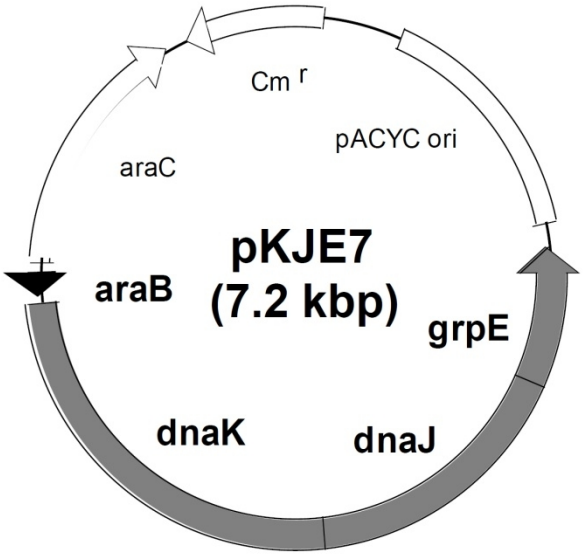

Figure 15: Chaperone plasmid pKJE7 (TaKaRa)

A preculture was prepared in LB medium supplemented with 100 mg/l ampicillin for the selection of pET-21b plasmid and 30 mg/l chloramphenicol for the selection of pKJE7 chaperone plasmid. The culture was incubated in a shaking water bath (GFL) at 30°C overnight. The expression culture was prepared using 2% (v/v) inoculum of the preculture in a fresh LB medium supplemented with 100 mg/l ampicillin, 30 mg/l chloramphenicol and 2 g/l L-arabinose for the expression of the chaperone proteins. The expression culture

was incubated in shaking water bath at 30°C until OD_{600} of 0.4-0.5. IPTG (0.5 mM) was added to induce the recombinant protein production. Culture was incubated in shaking water bath at 20°C for 5 hours. Cells were harvested by centrifugation (Sorvall Evolution Superspeed, rotor: SLC-6000, 7000 rpm, 15 min, 4°C). Cell pellet were stored at -20°C until used for preparation of cleared lysate.

Being in the insoluble fraction after the preparation of the cleared lysate, His-tagged PBPs were recovered and purified on Ni-NTA resin using sodium lauroylsarcosine as described in section 3.3.11.2.

The purified His-tagged PBPs were mixed immediately with 98% glycerol (1:1) and stored at -20°C.

3.3.11.4 Over-production and purification of His-tagged GlyA from *C. pneumoniae*

The plasmid pET-21b-*glyA* was transformed into *E. coli* BL21 (DE3). All the steps for over-production and protein purification are the same as described in section 3.3.11.1. All buffers (Table 16) used for purification were supplemented with 5 mM β-mercaptoethanol, 0.1 mM pyridoxal phosphate and 25% (w/v) glycerol to increase the stability of GlyA during purification.

At the end of purification steps, His-tagged GlyA elution fractions were stored directly at -20°C.

Table 16: Buffers used for the purification of His-tagged GlyA

Buffer	Composition
Lysis buffer	50 mM sodium phosphate buffer pH 7.5, 500 mM NaCl, 5 mM β-mercaptoethanol, 0.1 mM pyridoxal phosphate, 25% (w/v) glycerol
Wash buffer 1	50 mM sodium phosphate buffer pH 7.5, 500 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 0.1 mM pyridoxal phosphate, 25% (w/v) glycerol
Wash buffer 2	50 mM sodium phosphate buffer pH 7.5, 500 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 0.1 mM pyridoxal phosphate, 25% (w/v) glycerol

Buffer	Composition
Elution buffer	50 mM sodium phosphate buffer pH 7.5, 500 mM NaCl, 250 mM imidazole, 5 mM β-mercaptoethanol, 0.1 mM pyridoxal phosphate, 25% (w/v) glycerol

3.3.11.5 Purification of His/*Strep*-tagged GlyA from *C. pneumoniae*

The plasmid pET-52b-*glyA* was transformed into *E. coli* BL21 (DE3). All the steps for over-production are the same as described in section 3.3.11.1.

Protein was first purified based on its His-tag as described in section 3.3.11.4. Elution fractions containing the recombinant GlyA protein were pooled and loaded on a gravity flow column containing pre-equilibrated *Strep*-Tactin sepharose resin. Column was washed 5 times with 1 CV washing buffer (150 mM Tris/HCl pH 8, 150 mM NaCl, 5 mM β-mercaptoethanol, 0.1 mM pyridoxal phosphate, 25% glycerol). Protein was eluted with 3 CV washing buffer containing 2.5 mM desthiobiotin.

3.3.12 Protein buffer exchange

For the removal of salts, detergents and exchange of protein buffers, Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific) and Zeba™ spin desalting columns (Thermo Fisher Scientific) were used according to the manufacturer's instructions.

Slide-A-Lyzer is based on the principle of dialysis, in which small molecules and ions in solutions can pass through a semi-permeable membrane in the direction from high concentration to low concentration. On the other hand, high molecular weight molecules like proteins cannot pass through the membrane and remain within the cassette.

Zeba spin desalting columns are based on size exclusion chromatography resin, in which macromolecules as proteins can pass fast through the column while small molecules and ions are retained within the columns.

3.3.13 Analysis of the purified proteins

3.3.13.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a widely used technique to separate proteins according to their size using electric field. This can be achieved by using the strong anionic detergent sodium dodecyl sulfate (SDS) to linearize the protein by removing secondary and tertiary protein structures. Moreover, SDS uniformly covers the protein conferring it negative electric charge proportional to its size. Because charge per mass ratio is same among SDS-denatured protein, electrophoretic mobility of proteins in polyacrylamide gels will depend only on protein size or mass [128].

During this work, Pierce protein gels (Thermo Fisher Scientific) with 4-12% and 4-20% polyacrylamide gel gradient were used according to the manufacturer's instructions. Protein samples were first mixed with LDS sample buffer (4X). To break the possible disulfide bridges in the protein, dithiothritol (DTT) was added. The reaction mix was heated for 5 minutes at 95° in water bath (Julabo). For the analysis of separated proteins, Protein Marker Broad Range (NEB) and Novex Sharp Pre-stained Protein Standard (Invitrogen) were used during this work. Electrophoresis was performed using Novex Mini-Cell (Invitrogen) at 170 Volts for 55 minutes in Tris-HEPES-SDS running buffer. For staining of the gel after electrophoresis, PageBlue protein staining solution (Thermo Fisher Scientific) was used according to the manufacturer's instructions. The gel was first washed with distilled water for 3 times to remove SDS, and then stained with the protein staining solution for 1 hour until overnight with shaking. Finally, the stained gel was washed with distilled water.

3.3.13.2 Estimation of protein concentration

Estimation of protein concentration was achieved during this work using Bradford method [129]. This method involves the binding of Coomassie Brilliant Blue G-250 to proteins, which results in a shift in the absorption maximum of the dye from 365 nm to 595 nm and the increase in the absorption at 595 nm is directly proportional to the sample protein content.

During this work, protein estimation was performed in microtiter plates (VWR International). Protein samples or bovine serum albumin (BSA) (Sigma-Aldrich) standard solution (10 µl) were mixed with 40 µl Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories) and 160 µl deionized water. Samples were incubated for 10 minutes at room temperature and then absorbance at 595 nm was determined using microtiter plate reader NanoQuant (Tecan Group). For protein samples containing high concentrations of detergents, which are not compatible with the methods, protein samples were dialyzed or diluted to remove the detergents or their incompatibility.

3.4 BIOCHEMICAL METHODS

3.4.1 Nucleotide removal

To examine the effect of different types and concentration of ATP and GTP on the polymerization of chlamydial MreB, the already bound nucleotides were removed by treating the dialyzed MreB with Dowex resin as follow. Dialyzed MreB was mixed with 1/4th its volume with 50% Dowex resin (1X8-400Cl, Sigma-Aldrich) slurry in 10 mM Tris/HCl pH 8.0. The reaction was incubated on ice for 15 minutes with vortexing every 2-3 minutes, followed by centrifugation for 5 minutes (Heraeus Biofuge 28RS, rotor: 3751, 5300 rpm, 4°C). The supernatant was collected in a separate tube. This procedure was repeated 3 times to prove complete nucleotide removal.

3.4.2 MreB light scattering assays

MreB *in vitro* polymerization was investigated using light scattering assays modified from the one described by Bean an Amann for MreB of *Thermotoga maritima* (*T. maritima*) [130]. The standard polymerization reaction consisted of 5 µM MreB, 200 µM ATP, 5 mM $MgCl_2$, 1 mM EGTA, and 10 mM imidazole buffer, pH 7 in a final volume of 800 µl. The non-protein components were mixed. Separately on ice, MreB was mixed with 1/9th volume of 10X cation exchange buffer (1 mM $MgCl_2$, 10 mM EGTA) and incubated for 1 minute. Polymerization reaction was initiated by mixing the two solutions together and detected by quantifying the decreases in the percentage of transmission at 400 nm using Nanophotometer (Implen) at room temperature for 15 minutes. All experiments were

performed in triplicate. For A22 inhibition studies, A22 (Merck) was dissolved in DMSO (Sigma-Aldrich) and added to the non-protein part of the polymerization reaction in a final concentration of 5, 50 and 300 µM.

3.4.3 MreB sedimentation assays

Experiments followed the protocol of Bean and Amann [130]. MreB (5 µM) was polymerized under standard conditions in presence or absence of 300 µM A22 for 1 hour at 20°C. Polymerized MreB was centrifuged (Heraeus Biofuge 28RS, rotor: 3753, 22000 rpm, 49.500 g, 30 minutes 20°C). The pellet was washed twice with the polymerization buffer followed by centrifugation. Finally, equivalent volumes of pellet and supernatant were analyzed by SDS-PAGE and Coomassie Blue staining.

3.4.4 MreB and MurF co-pelleting assays

In vitro interaction of MreB with MurF was investigated following a previously described co-pelleting protocol [131]. The sedimentation assays in section 3.4.3 were performed with MreB (5 µM) polymerized in presence of different concentrations chlamydial MurF (2.5, 5, 10 and 15 µM). Reactions were incubated for 1 hour at 30°C in a water bath. As a control, MurF (15 µM) was incubated in the reaction buffer without MreB to ensure the non-specific sedimentation of MurF. In another control, MreB (5 µM) was allowed to polymerize in presence of BSA (15 mM) to ensure the non-specific interaction of MreB with proteins other than MurF. At the end of incubation time, samples were centrifuged (Heraeus Biofuge 28RS, rotor: 3753, 22000 rpm, 49,500g, 30 minutes 30°C), washed and analyzed using SDS-PAGE and Coomassie Blue.

3.4.5 Phosphate release assays

To analyse ATPase activity of MreB, phosphate release using a molybdate:malachite green assay was investigated. The technique is based on the formation of complex between malachite green and phosphomolybdate under acidic conditions.

Wild-type and C345A mutant MreB were incubated with ATP (100 µM) using standard polymerizing and non-polymerizing (in the absence of $MgCl_2$, pH 8) conditions. Control reactions without ATP and without MreB were also performed. At different time

intervals, 100 µl aliquots were removed and mixed directly with 5 µl ice cold 70% perchloric acid (Merck) to stop the enzymatic reaction. The reactions were then incubated on ice for 10 minutes and centrifuged for 5 minutes at 49,500g, 4°C (Heraeus Biofuge 28RS). The supernatant was mixed with equal volume of Molybdate Dye Solution (Promega) and incubated in microtiter plate for 30 minutes at room temperature. Absorbance was measured at 600 nm in a microtiter plate reader NanoQuant (Tecan Group). All experiments were carried out in triplicate.

3.4.6 Detection of racemization activity of chlamydial GlyA

To investigate the *in vitro* racemization activity of chlamydial GlyA, a previously described racemization reaction for GlyA from *E. coli* was performed [98]. GlyA dialyzed against 20 mM BES buffer pH 7.0 (Sigma-Aldrich) containing 0.1 mM pyridoxal phosphate was mixed in 40 µl reaction volume with 25 mM L-alanine or 1 mM D-alanine, 0.03 mM pyridoxal phosphate and 1 mM DTT in 50 mM BES buffer pH 7.5. The reaction was incubated at 37°C overnight. Control reactions without amino acid or without GlyA were also performed.

D-alanine produced or consumed by the expected racemization activity on L- or D-alanine respectively were detected using a fluorescent-coupled enzyme assay [132]. This assay based on oxidizing D-alanine into pyruvate, ammonia and hydrogen peroxide using the activity of D-amino acid oxidase (DAO) followed by quantitative detection of released hydrogen peroxide using fluorescent substrate QuantaBlu. Its oxidized form has an excitation wavelength of 325 nm and emission wavelength of 420 nm.

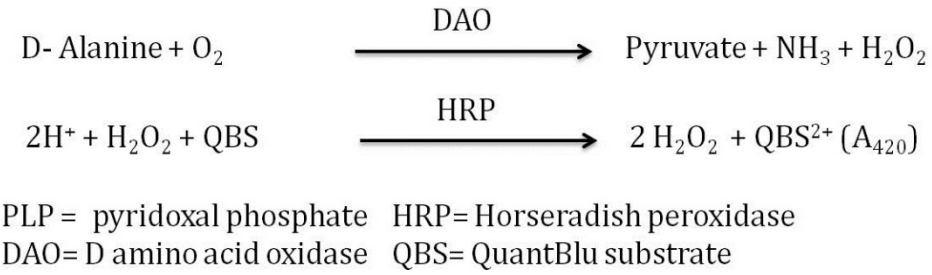

Figure 16: Fluorescent-coupled enzyme assay reactions for detection of D-alanine [132]

A detection buffer for D-alanine was prepared containing 10 µl QuantaBlu substrate (Thermo Fisher Scientific), 2.78 µg/ml FAD, 1 unit horseradish peroxidase, 0.06 unit DAO, 100 mM Tris/HCl pH 8.5 in 360 µl final volume. Each racemization reaction was mixed with 360 µl detection buffer and incubated for 1 hour at room temperature. Samples were then analyzed using fluorescent spectrophotometer at excitation wavelength 325 nm and emission wavelength 420 nm.

3.4.7 Butanol/pyridine acetate extraction of lipids

Lipids can be extracted from reaction mixtures with the help of n-butanol/pyridine acetate [133]. During this work, butanol/pyridine acetate pH 4.2 was added to equal volume of reaction samples containing lipids. The reaction was vortexed for 1 minute using Vortex Genie (Scientific Industries) and then centrifuged (Eppendorf MiniSpin, 13400 rpm, 5 minutes) to separate aqueous and organic phases. The upper phase containing lipids was then analyzed using thin layer chromatography (TLC).

3.4.8 Thin layer chromatography for detection of lipids

Thin layer chromatography (TLC) can be used for the analysis of lipid extracts. It is a separation process, in which lipids are separated according to their different solubilities in the mobile and stationary phases. The lipids are transported by a mobile phase on a silica gel plate (stationary phase). The mobile phase is a non-polar solvent, which moves by capillary action along the stationary phase. How far the lipids from the mobile phase can be taken along depends on the polarity of the lipids and their interaction with the silica gel. The stronger the interaction of lipids with the mobile phase, the further they are transported.

During this work, silica gel plates 60 with 0.25 mm layer thickness (Merck) were used. The lipid extracts were spotted onto the plate and dried. The loaded TLC plate was placed in a glass chamber (Desaga) with a mobile phase (Table 17) according to the recipe by Rick *et al*. [134]. The chromatography was allowed to run for 30-50 minutes then the TLC was removed and air-dried. To visualize the lipid bands, the TLC plate was immersed in a phosphomolybdic acid staining solution (PMA) (Table 17) to cover the entire surface,

and then dried. The silica gel plate was developed on a precision hotplate (Harry & Gestigkeit IKA-Werke) at 130°C afterwards.

Table 17: Solutions used for thin layer chromatography

Solution	Content
Mobile phase	Chloroform, methanol, distilled water., ammonium-hydroxide [88:48:10:1 (v/v)]
Phosphomolybdic acid (PMA) solution	2,5 % (w/v) phosphomolybdic acid and 1% (w/v) ceric-sulfate in 6 % (w/v) sulfuric acid

3.4.9 Testing the transpeptidase activity of chlamydial PBP2 and PBP3

3.4.9.1 Preparation of peptidoglycan sacculi from *E. coli*

In a transpeptidation reaction catalyzed by PBPs, the newly synthesized cell wall materials are attached to cell wall via a peptide bond. Therefore, to test the transpeptidase activity of the chlamydial PBP2 and PBP3 using their natural substrates, both the cell wall precursor, lipid II and PG sacculi should be used in the assay. Two types of PG sacculi of different bacterial sources were used; PG sacculi from *B. subtilis* (Sigma-Aldrich) and from *E. coli* W3110 (prepared during this work).

LB medium (1 liter) was inoculated with 1% (v/v) *E. coli* W3110 preculture and incubated at 37°C until OD_{600} of 0.5-0.6. Cells were harvested by centrifugation (Sorvall Evolution Superspeed, rotor: SLC-6000, 7000 rpm, 15 min, 4°C). The pellet was resuspended in 20 ml PBS buffer (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4 $2H_2O$, 0.24 g/l KH_2PO_4) and poured on 80 ml of boiling SDS solution (5% w/v) with stirring. The mixture is allowed to boil for 30 minutes then incubated at room temperature overnight to allow complete cell lysis and release of murein sacculi. Separation of the sacculi was achieved by centrifugation (Heraeus, rotor: 3764, 13500 rpm, 30 minutes, 25°C). The pellet was washed 3-4 times with distilled water to remove SDS. The pellet is then resuspended in 1 ml PBS buffer and digested overnight at 37°C with α-chymotrypsin (300 µg/ml final concentration) to break down proteins. On the next day, another 300 µg/ml were added and reaction was

incubated for 3 hours at 37°C. *E. coli* sacculi were separated by centrifugation and washed 3-4 times with distilled water. Finally, the pellet was resuspended in 100 µl PBS buffer and stored at -20°C.

3.4.9.2 Testing transpeptidase activity using natural substrates, lipid II and PG sacculi

Born *et al.* [106] showed that transpeptidase domain of bi-functional PBP1a from *E. coli* is not able to show *in vitro* activity in absence of a functional transglycosylase domain. Based on these results, mono-functional transpeptidases such as PBP2 and PBP3 from *C. pneumoniae* cannot exhibit activity disconnected from transglycosylation. For this reason PBP2 mutant from *Staphylococcus aureus,* a bi-functional PBP, with a functional transglycosylase domain and a mutated dysfunctional transpeptidase domain, was added to the assay.

PBP2 or PBP3 from *C. pneumoniae* together with PBP2 mutant from *S. aureus* were mixed with PG sacculus in a buffer containing 25 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 0.05% Triton X-100. The reaction volume was adjusted to 30 µl and incubated on ice for 10 minutes. Lipid II (2 nmol dissolved in 0.05% Triton X-100) was added and the reaction was incubated at 30°C for 2 hours. Samples were centrifuged (Eppendorf MiniSpin, 13400 rpm, 10 minutes) to remove the PG sacculus and lipid II in the supernatant was detected by TLC.

3.4.9.3 Testing transpeptidase activity using artificial thioester S2d substrate

Thioester analogs of D-alanyl-D-alanine moiety of the bacterial cell wall stem peptide have long been used to study the transpeptidation reactions catalyzed by penicillin binding proteins [135-137]. The use of such chromogenic substrates facilitates the detection of the reaction by measuring the absorbance at 254 nm. Coupling of the released product to 4,4'-dithiopyridine increases the sensitivity of the assay by 15 folds. In presence of 4,4'-dithiopyridine, the increase of the reaction absorbance at 325 nm is used as a measure for the transpeptidase activity.

The thioester N-benzoyl-D-alanylmercaptoacetic acid (S2d) (Figure 17) was supplied kindly by Prof. Dr. Piel (University of Bonn, Germany). A stock solution of S2d substrate was prepared by dissolving the substrate in 100 µL DMSO, then add 10 mM sodium phosphate buffer pH 7 to reach the final concentration (10 mM). PBPs were dialyzed against 10 mM sodium phosphate buffer pH 7.0 or 10 mM tris buffer pH 7.5 containing 10% DMSO (v/v) and 500 mM NaCl. PBP2 or PBP3 from *C. pneumoniae* was mixed with 1 mM S2d and 0.8 mM 4,4'-dithiopyridine in presence or absence of DMSO (10% v/v). The reaction was incubated at 37°C for 1 hour with absorbance at 325 nm measured every 10 minutes using Nanophotometer (Implen).

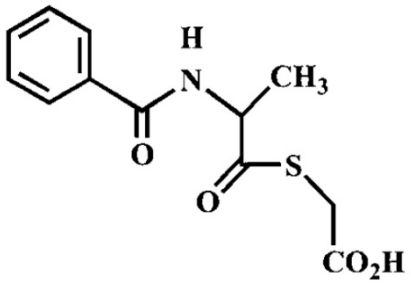

Figure 17: Chemical structure of the thioester N-benzoyl-D-alanylmercaptoacetic acid (S2d).

3.4.10 Testing the DD-carboxypeptidase activity of chlamydial PBP6a

DD-carboxypeptidases are capable of removing the terminal D-alanine from the stem peptide of PG resulting in tetrapeptide side chain which cannot be cross-linked by transpeptidases [104]. To detect the DD-carboxypeptidase activity of PBP6a, two different substrates were tested.

3.4.10.1 Lipid II as a substrate for PBP6a

In this assay, PBP6a (1 µg) was added to 2 nmol of lipid II dissolved in 0.1% triton in 30 µl reaction containing 100 mM NaCl, and 50 mM Tris/HCl pH 7.5. The reaction was incubated 3 hours or overnight. At the end of the incubation time, the reaction lipid content was analyzed by TLC. The release of the terminal D-alanine was expected to show a difference in the mobility during TLC analysis.

3.4.10.2 Nα,Nε-diacetyl-L-lys-D-ala-D-ala as a substrate for PBP6a

This assay was described previously by Gutheil *et al.* [132]. PBP6a (1 µg) was mixed with 1 mM of the artificial substrate Nα,Nε-diacetyl-L-lys-D-ala-D-ala (Sigma-Aldrich) in 40 µl of 100 mM Tris/HCl pH 7.5, 0.5 mg/ml BSA, 100 mM NaCl and 150 pmol D-alanine. The reaction was incubated at 30°C for 3 hours overnight. The released D-alanine was detected using the fluorescent-coupled enzyme assay described in section 3.4.6

3.5 BACTERIAL ADENYLATE CYCLASE TWO-HYBRID SYSTEM ASSAYS

The bacterial adenylate cyclase two-hybrid system (BACTH) allows the *in vivo* investigation of protein-protein interactions. The principle of the assay based on the interaction mediated reconstitution of active adenylate cyclase enzyme [138]. The catalytic domain of the adenylate cyclase enzyme from *Bordetella pertussis* consists of two fragments, T25 and T18 [139]. When both fragments are separated, the protein is not active. Fusion of the two fragments to two interacting proteins results in a functional complementation between T25 and T18 fragments and, therefore, cAMP is produced. Binding of the latter to the catabolite activator protein (CAP) regulates gene transcription in *E. coli*. Genes of *lac* and *mal* operons are expressed and higher β-galactosidase activity can be detected.

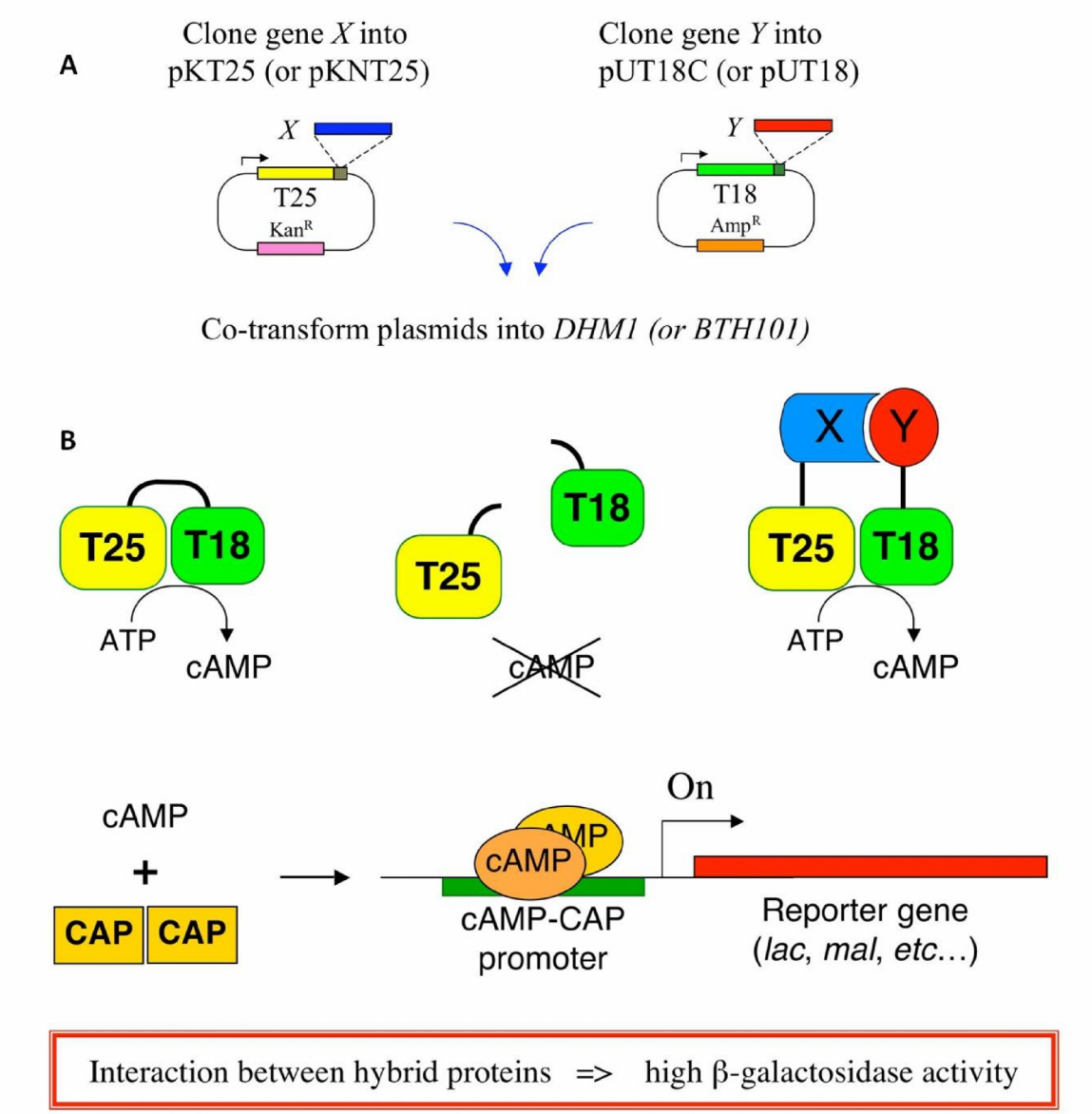

Figure 18: Schematic presentation of BACTH system.

(A) The recombinant plasmids constructed to fuse the T25 and T18 to test proteins. (B) The principle of BACTH system. CAP = catabolite activator protein.

3.5.1 Detection of BACTH system protein-protein interaction using β-galactosidase assay

During BACTH system assays, protein-protein interaction is associated with increase in the β-galactosidase activity, which can be measured quantitatively using a β-galactosidase assay.

Buffers and solutions required for the β-galactosidase assay are listed in Table 18. The two recombinant BACTH plasmids pKNT25 (or pKT25) and pUT18 (or pUT18C) were co-transformed into adenylate cyclase deficient *E. coli* strain BTH101. Empty BACTH vectors and pKT25zip/pUT18Czip were co-transformed in the same strains as negative and positive controls respectively. Three different clones of each transformation were used to test the β-galactosidase activity. Each clone was used to infect 3 ml of the minimal medium M63 containing 25 µg/ml kanamycin and 50 µg/ml ampicillin to prepare the precultures that were incubated at 37°C overnight in a rotary incubator (Heraeus). The precultures (30 µl) were used to infect another 3 ml of M63 medium containing 0.5 mM IPTG, 25 µg/ml kanamycin and 50 µg/ml ampicillin. The cultures were incubated under the same conditions. Daily, 350 µl aliquots were taken and incubated on ice for 30 minutes then diluted with 1400 µl M63 medium. The OD_{600} of the diluted cultures was measured and sodium lauroylsarcosine (2% end concentration) was added to the rest of the diluted cultures and incubated at 30°C to induce cell lysis. The reaction was diluted with equal volume of Z buffer (Table 18) and incubated at 30°C for 5 minutes. To 1 ml of the diluted reaction, 250 µl O-nitrophenyl-β-D-galactoside (ONPG) was added. β-galactosidase hydrolyzed the synthetic substrate ONPG with the production of the yellow colored product O-nitrophenol. The reaction was stopped with 500 µl stop solution and absorbance at 420 nm was measured. When ONPG is in excess, the production of o-nitrophenol per unit time is proportional to the activity of β-galactosidase; thus, the production of yellow color can be used to quantify the enzyme activity, which is expressed as Miller units calculated with the following formula:

$$Miller\ units = \frac{1000 * A_{420}}{T * V * OD_{600}}$$

T = time between addition of ONPG and reaction stop

V = culture volume in ml

Table 18: Solutions required for β-galactosidase assay

Solution	Content
M63 minimal medium	2 g/l $(NH_4)_2SO_4$, 13.4 g/l KH_2PO_4, 0.5 mg/l $FeSO_4$ $7H_2O$, pH 7. After autoclaving, filter sterilized 1 ml $MgSO_4$ (1M), 10 ml glucose (20% w/v) and 2 ml thiamine HCl (0.05%) are added
Z buffer	0,06 M $Na_2HPO_4 \cdot 2H_2O$, 0.04 M $NaH_2PO_4 \cdot 2H_2O$, 0.01 M KCl, M 0.001 M $MgSO_4$
ONPG solution	4 mg/ml in distilled water
Stop solution	1 M Na_2CO_3

4 RESULTS

4.1 FUNCTIONAL ANALYSIS OF THE CHLAMYDIAL ACTIN HOMOLOG MREB

All bacteria in the family *Chlamydiaceae* lack the essential cell division protein FtsZ, but harbor, despite of their spherical shape, the actin homolog MreB. The role of the cytoskeletal protein in such endobacteria is not known. It was shown recently that maintaining lipid II biosynthesis in cell wall-less bacteria reflects an essential role of the lipid II biosynthesis pathway for prokaryotic cell division [95]. MreB could be involved in maintaining coordinated cell division via tethering lipid II biosynthesis to the chlamydial septum.

4.1.1 Cloning, over-production and purification of chlamydial MreB

The *mreB* gene from *C. pneumoniae* was cloned in the expression vectors, pASK-IBA3 and pET-21b (done by Christian Otten). The pASK-IBA3-*mreB* construct introduces a C-terminal *Strep*-tag, while the pET-21b-*mreB* one fuses a C-terminal His-tag (Figure 19)

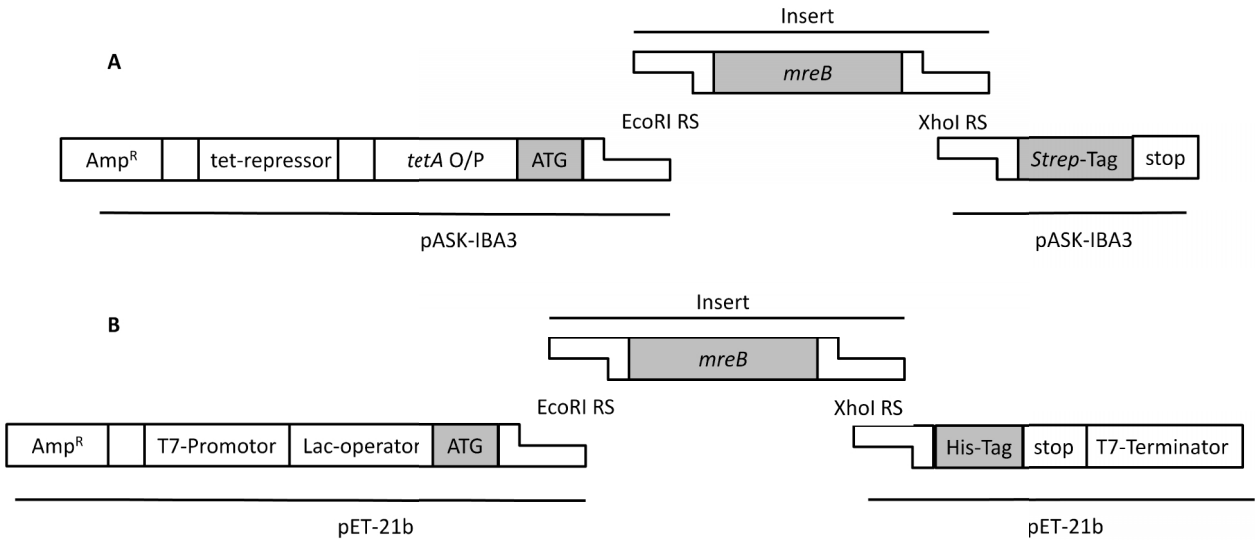

Figure 19: Schematic presentation of the recombinant plasmids pASK-IBA3-*mreB* (A) and pET-21b-*mreB* (B)

4.1.2 Over-production and purification of chlamydial MreB

The *Strep*-tagged MreB was purified from 1 liter culture after overnight induction using 200 ng/ml anhydrotetracycline. The cleared lysate was loaded on a *Strep*-Tactin column. After washing steps, *Strep*-tagged protein was eluted with a buffer containing 2.5 mM desthiobiotin (section 3.3.10.1). The over-production of His-tagged MreB was induced with 0.5 mM IPTG for 4-5 hours. The cleared lysate was purified on Ni-NTA column. The His-tagged protein was eluted with a buffer containing high concentration of imidazole (200-250 mM) (section 3.3.11.1).

In this project, both MreB-*Strep* and MreB-His_6 were successfully purified (Figure 20) but the purity and the overall yield of the MreB-His_6 were significantly higher, the latter may be a result from different strengths of the *tetA* and T7 promoters. Hence, the recombinant His-tagged protein was used to analyze the activity of MreB from *C. pneumoniae*.

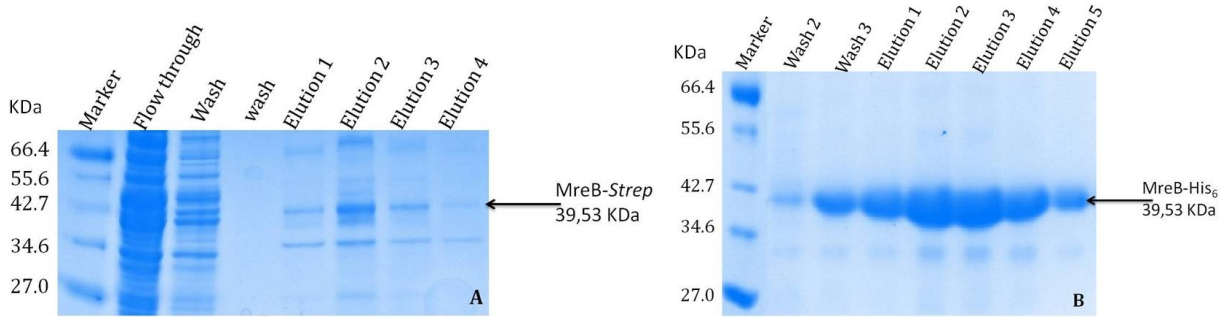

Figure 20: SDS-PAGE of purification of MreB-*Strep* (A) and MreB-His_6 (B)

Marker: protein marker (Broad Range 2-212 KDa, NEB)

4.1.3 Investigation of chlamydial MreB polymerization *in vitro*

To examine the functionality of MreB, the ability of the protein to polymerize *in vitro* was tested under different conditions of pH, Mg^{2+} concentration and K^+ concentration as done recently for MreB from *T. maritima* (MreB1) [130] and *B. subtilis* [140].

Chlamydial MreB polymerized *in vitro* in light scattering (Figure 21) and sedimentation assays (Figure 22 B). The rate and extent of polymerization were favored at slightly acidic pH values and in presence of Mg^{2+} ions but inhibited by K^+ ions (Figure 21 A-

C). The chlamydial protein showed more similarity to MreB from *B. subtilis* than to MreB from *T. maritima* regarding its pH and K^+ dependence. Polymerization of MreB from *C. pneumoniae* and *B. subtilis* was greatly impaired or completely inhibited at pH values ≥ 8 and strongly inhibited at KCl concentrations of 20 mM or higher [140]. In contrast, MreB from *T. maritima* was shown to polymerize at pH as high as 9.5 and in presence of 20 mM KCl [130]. These properties probably reflect the adaptation of this extremophilic organism to geothermally heated marine sediments.

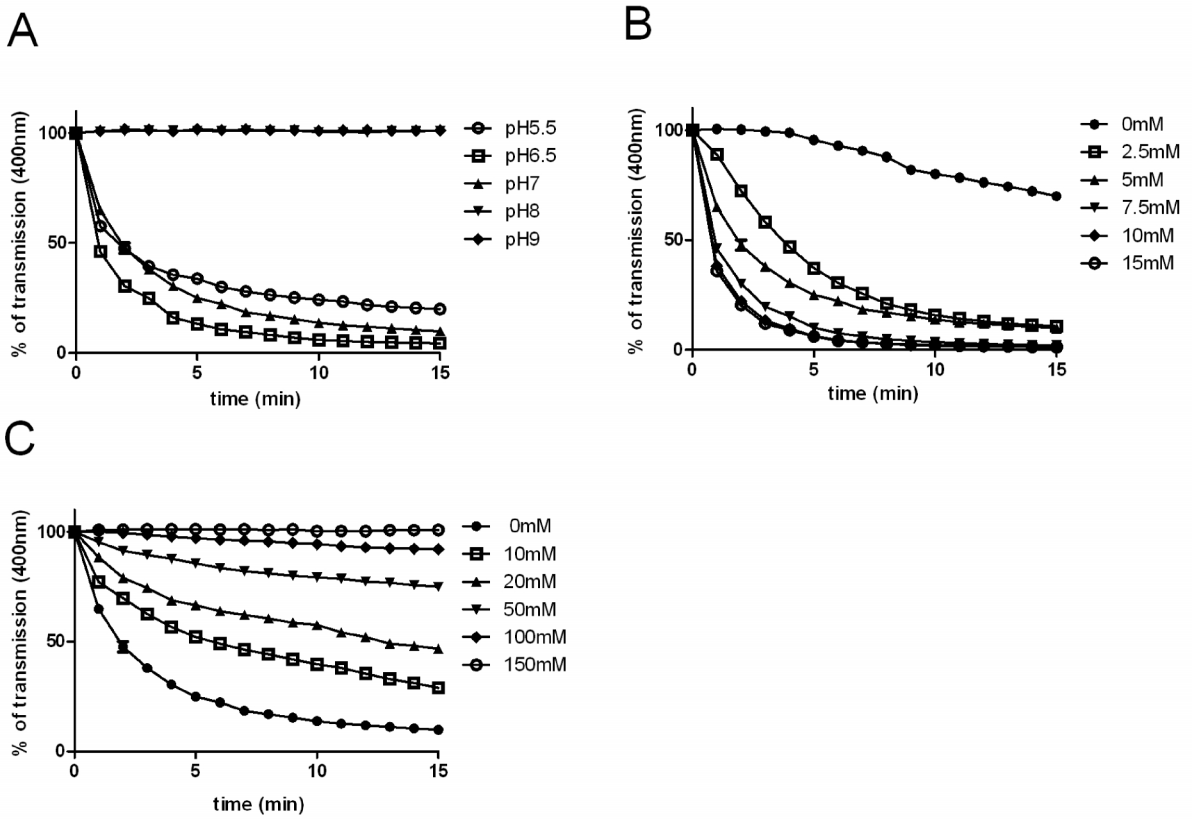

Figure 21: Polymerization of wild-type MreB from *C. pneumoniae*.

In vitro light scattering assays were performed at varying pH (A) $MgCl_2$ (B) and KCl (C) concentrations using 5 µM chlamydial MreB

4.1.4 Investigation of the effect of S-(3,4-dichlorobenzyl) isothiourea (A22) on the *in vitro* polymerization of MreB

S-(3,4-dichlorobenzyl)isothiourea (A22) was firstly defined as a compound that induces spherical cells in *E. coli* [141]. A22 is now used to disrupt the cell cycle-related

reorganization of the MreB cytoskeleton *in vivo* causing its disassembly with consequent loss of rod shape and cell death [68, 142]. The compound impairs the ATP dependant polymerization of *T. maritima* MreB *in vitro* by competitive binding into the nucleotide-binding pocket of the protein [143].

The effect of A22 on the *in vitro* polymerization of MreB form *C. pneumoniae* was investigated using light scattering and sedimentation assays. Both experiments revealed that A22 did not impair the polymerization of chlamydial MreB *in vitro* (Figure 22).

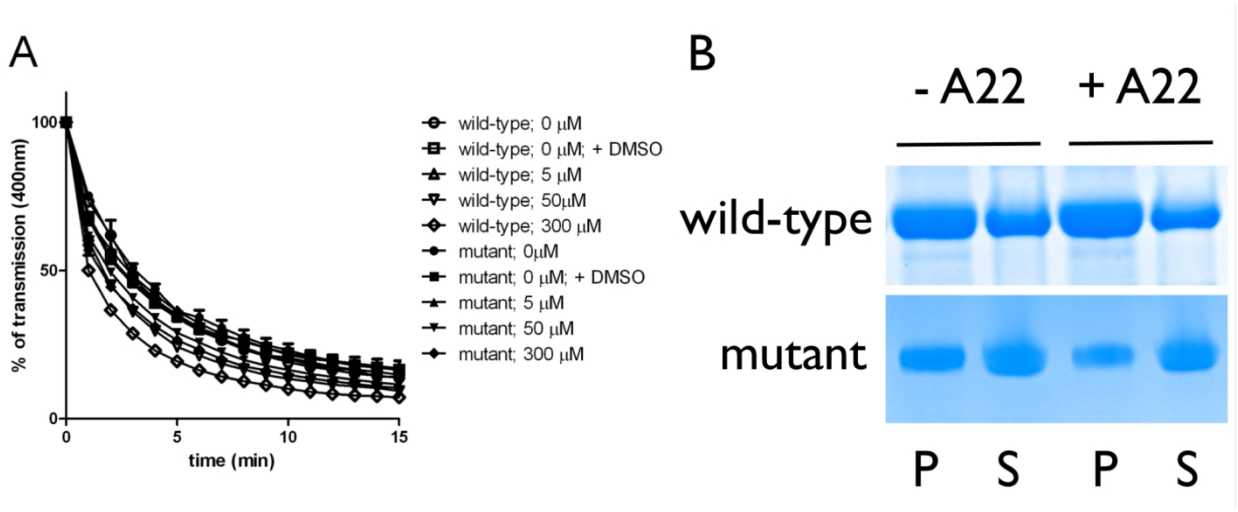

Figure 22: Influence of A22 on the polymerization of chlamydial MreB.

The effect of A22 on MreB wild-type and C345A mutant polymerization was determined by light scattering (A) and sedimentation assays (B). For sedimentation assays, polymerized MreB was centrifuged and equivalent volumes of pellet (P) and supernatant (S) were separated by SDS-PAGE

4.1.5 Preparation of C345A MreB mutant

In a previous study [68], A22 selection pressure was used to generate seven A22-resistant stable mutants from *C. crescentus*. Sequence analysis of all mutants showed amino acid exchanges in MreB in residues that were conserved in MreB from *C. crescentus*, *T. maritima*, and *E. coli* (Figure 23A). Five of the seven exchanges were located in the nucleotide-binding pocket and the last two were present in a helix that contacts the nucleotide. Interestingly, one of these altered residues in mutant *C. crescentus* MreB is intrinsically exchanged in wild-type MreB from *C. pneumoniae* (Ala345 located in helix 12 is replaced by cysteine, numbering of *C. pneumoniae*) (Figure 23B).

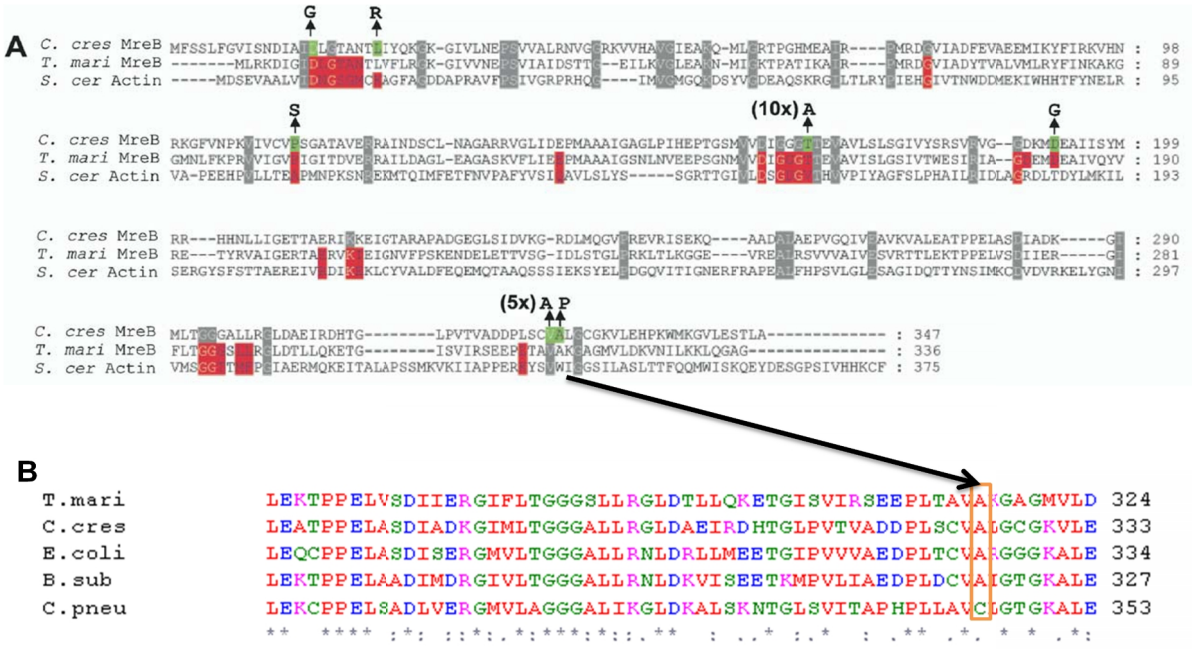

Figure 23: Schematic presentation of amino acid exchange map in MreB from A22-resistant *C. crescentus* mutants (A) [68] and intrinsically exchanged residue 345 in MreB from *C. pneumoniae* (B).

To test whether this natural alteration in chlamydial MreB is responsible for resistance to A22, Cys345 was replaced with alanine to restore the conserved residue in *C. crescentus*, *T. maritima*, *B. subtilis* and *E. coli*. With the help of site-directed mutagenesis, C345A MreB mutant was successfully prepared and the desired mutation was confirmed by sequencing.

4.1.6 Over-production and purification of C345A MreB mutant

C345A MreB-His_6 mutant was successfully over-produced and purified using the same protocol described for the wild-type MreB-His_6 (Figure 24).

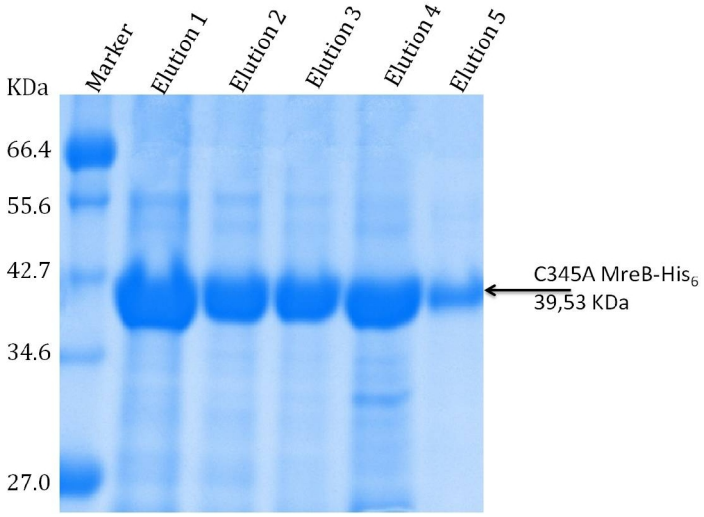

Figure 24: SDS-PAGE of C345A MreB-His_6 mutant purification from *C. pneumoniae*.
Marker: protein marker (Broad Range 2-212 KDa, NEB)

4.1.7 Studies on the effect of A22 on the *in vitro* polymerization of C345A MreB mutant

To examine whether this intrinsic exchange of amino acid residue 345 in wild-type MreB is responsible for its resistance to A22, the effect of A22 on the *in vitro* polymerization of C345A MreB was investigated using light scattering and sedimentation assays. Interestingly, the C345A mutant showed the same phenotype like the wild-type MreB protein and its polymerization was not affected by A22 (Figure 22).

4.1.8 Detection of the effect of nucleotides on MreB wild-type and C345A mutant polymerization *in vitro*

To further analyze the resistance of chlamydial MreB to A22, the capability of wild-type and C345A MreB mutant to polymerize in the absence of nucleotides was investigated (Figure 25).

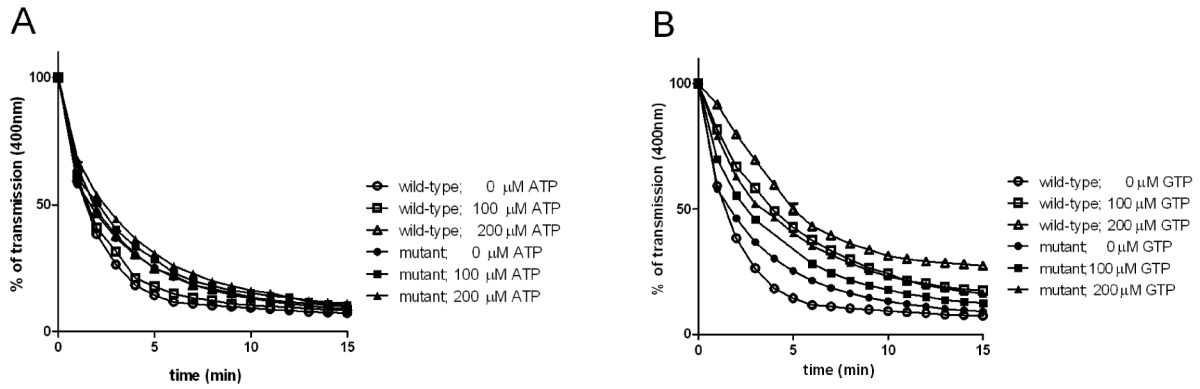

Figure 25: Influence of nucleotides on MreB polymerization.

Polymerization of chlamydial wild-type and C345A mutant MreB was investigated in the presence and absence of ATP (A) and GTP (B) using light scattering assays under standard conditions.

Remarkably, both the rate and extent of polymerization of chlamydial MreB wild type and mutant were nearly similar in the absence or presence of ATP (Figure 25A). On the other hand, the polymerization of chlamydial MreB in presence of GTP was impaired concerning its extent and kinetics. Binding of GTP to the nucleotide-binding pocket of chlamydial MreB might introduce conformational changes that affect the polymerization process.

To exclude that MreB-bound, co-purified ATP might have overcome the need to add external ATP to drive the polymerization reaction, MreB wild type and mutant were treated with an anion exchange resin prior to use, as described previously for nucleotide exchange procedures for MreB [130, 140]. The so treated MreB was still capable of polymerization without the addition of ATP (Figure 26).

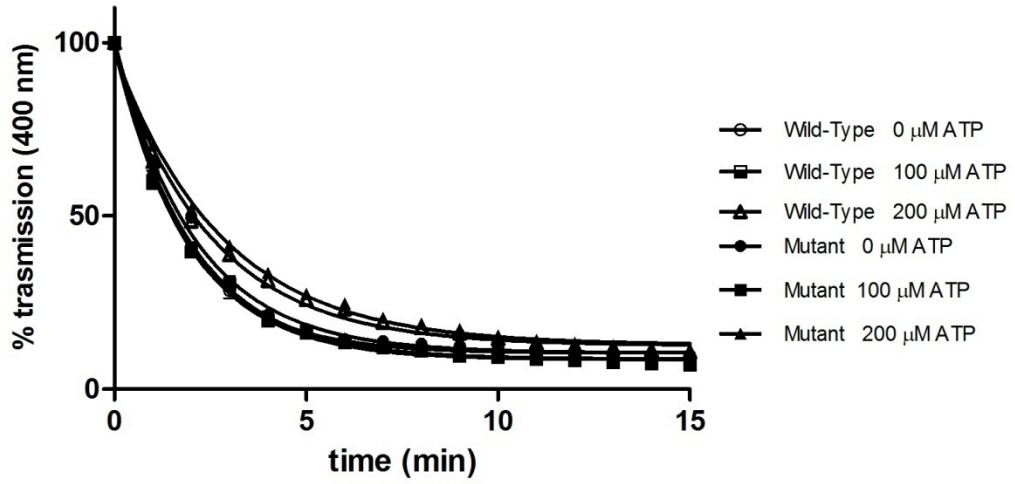

Figure 26: Influence of ATP on MreB polymerization after anion exchange treatment.

Polymerization of wild-type and C345A mutant MreB was investigated after the removal of bound nucleotides using Dowex resin.

Therefore, it appears reasonable that a competitive inhibitor of nucleotide binding such as A22 cannot block the polymerization of chlamydial MreB proteins. These findings concerning ATP independent polymerization are consistent with previous *in vitro* results on MreB from *B. subtilis* which showed that the purified actin homolog polymerized in a nucleotide independent fashion [140].

4.1.9 Detection of ATPase activity of wild-type MreB and C345A mutant *in vitro*

Although the polymerization of MreB from *B. subtilis* is ATP independent, the protein has ATPase activity [140]. To test whether chlamydial MreB can hydrolyze ATP, phosphate release assays were performed under monomeric and polymeric conditions (Figure 27). Both C345A mutant and wild-type MreB proteins hydrolyzed ATP without marked differences. Applying conditions that hinder MreB polymerization (pH 8.0 and absence of Mg^{2+}), monomeric MreB hydrolyzed ATP continuously during the whole time of the experiment (1 hour). On the other hand, under standard polymerization conditions (pH 7.0, Mg^{2+} 5 mM), ATPase activity was stalled once polymerization was completed (after 15 minutes). Additionally, under non-polymerizing conditions MreB released approximately 3

times more phosphate compared to polymerized MreB. These results indicate that ATPase activity is associated with the monomeric stage of MreB.

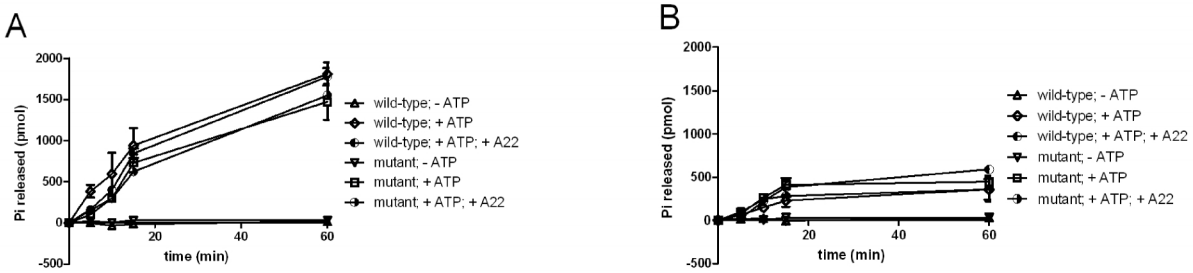

Figure 27: ATPase activity of wild-type and C345A mutant MreB under monomeric (A) and polymeric (B) conditions.

To check if A22 affects the ATPase activity of MreB, A22 was included in assays under polymerizing and non-polymerizing conditions. A22 showed no inhibitory effect on ATP hydrolysis in all experiments. This indicates that the compound cannot bind to the nucleotide-binding pocket of both wild-type and C345A mutant MreB.

4.1.10 Investigation of MreB interactions with MurF, MurG and MraY

In order to understand the role of MreB in cell wall biosynthesis, its interactions with MurF, MurG and MraY, three key enzymes in lipid II biosynthesis, were examined. The ligase MurF attaches the dipeptide D-ala-D-ala producing MurNAc-pentapeptide. The latter is transferred to the membrane lipid carrier C_{55}-P forming lipid I through the activity of MraY. Subsequently, MurG adds N-acetylglucosamine to lipid I yielding the final peptidoglycan precursor lipid II.

4.1.10.1 Cloning, over-production and purification of chlamydial MurF

4.1.10.1.1 Cloning of *murF* from *C. pneumoniae* in pASK-IBA5 and pET-21b vectors

The *murF* gene was cloned in pASK-IBA5 expression vector to construct N-terminal *Strep*-tagged MurF fusion protein (MurF-*Strep*) and in pET-21b vector to construct a C-terminal His_6-tagged MurF fusion protein (His_6-MurF) (Figure 28).

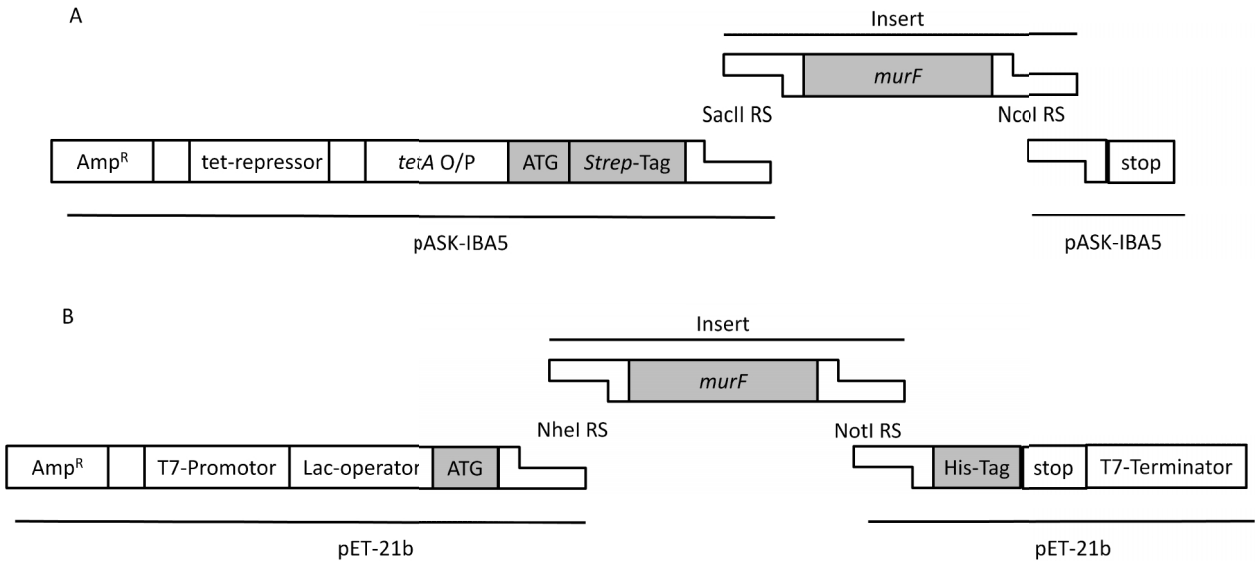

Figure 28: Schematic presentation of the recombinant plasmids pASK-IBA5-*murF* (A) and pET-21b-*murF* (B).

RS = restriction site.O/P = operator/promoter

The constructed plasmids were transformed into chemo-competent DH5α-*E. coli* strain. Positive clones were selected by colony PCR (Figure 29) and correctness of the inserted gene was confirmed by sequencing.

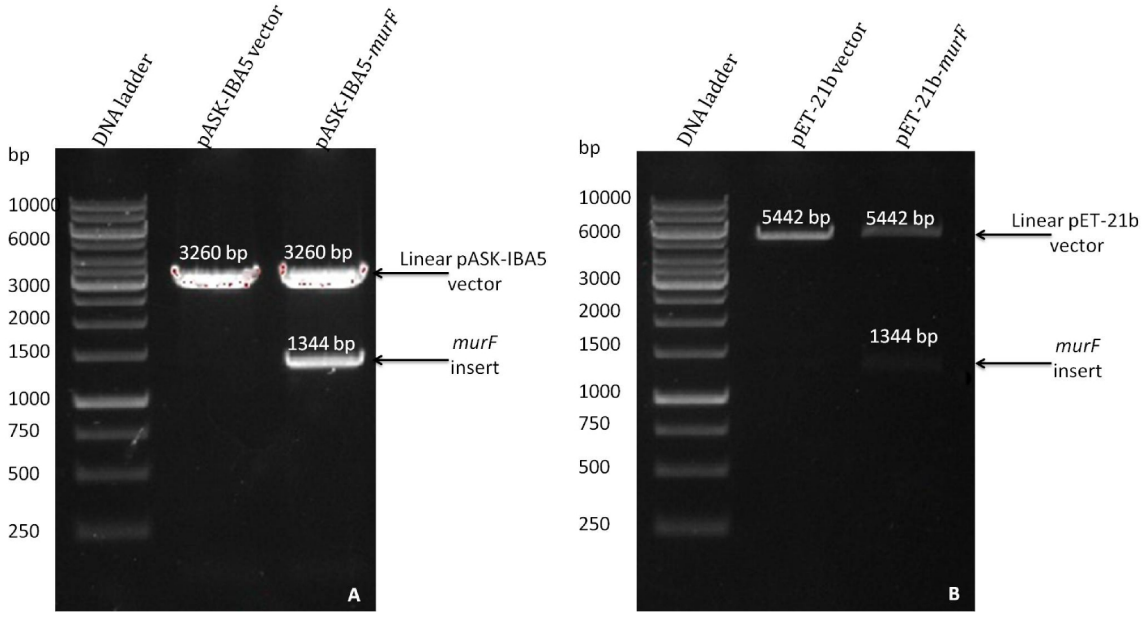

Figure 29: Detection of positive clones from recombinant plasmids pASK-IBA5-*murF* (A) and pET-21b-*murF* (B) using restriction analysis and agarose gel electrophoresis.

One mg of plasmid DNA was restricted for 1 hour with SacII/NcoI (A) or NheI/NotI (B). DNA ladder: 1 Kb DNA ladder (Fermentas)

4.1.10.1.2 Over-production and purification of chlamydial MurF

Strep-tagged MurF was purified from 1 liter culture after overnight induction using 200 ng/ml anhydrotetracycline. The cleared lysate was loaded on *Strep*-Tactin columns. After washing steps, the *Strep*-tagged protein was eluted with a buffer containing 2.5 mM desthiobiotin (section 3.3.10.1). His-tagged MurF was purified from 1 liter culture after induction (4-5 hours) with 0.5 mM IPTG. The cleared lysate, prepared with the help of 0.5% (w/v) sodium lauroylsarcosine to solubilize inclusion bodies, was loaded on Ni-NTA column. After washing steps, the His-tagged protein was eluted with a buffer containing high concentration of imidazole (200 mM) (sections 3.3.11.1 and 3.3.11.2).

During this work, both *Strep*-MurF and MurF-His_6 were successfully purified. The purity of the *Strep*-tagged protein was better but the yield of the MurF-His_6 was significantly higher (Figure 30). Therefore, the MurF-His_6 was used in the interaction assays.

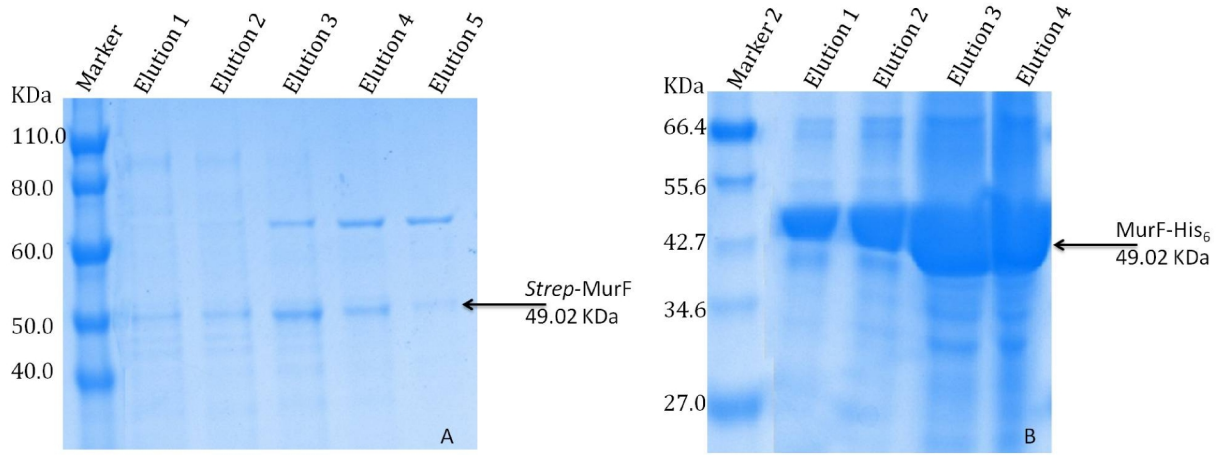

Figure 30: SDS-PAGE showing purification of *Strep*-MurF (A) and MurF-His_6 (B) from *C. pneumoniae*.

Marker: protein marker (Broad Range 2-212 KDa, NEB)

Marker 2: Novex Sharp Pre-stained Protein Marker (Invitrogen)

4.1.10.2 Investigation of chlamydial MreB interaction with MurF *in vitro*

MreB was tested for interactions with the cytoplasmic ligase MurF using a co-pelleting assay. MreB was polymerized in presence of the MurF. After centrifugation, equivalent volumes of the pellet and supernatant were analyzed using SDS-PAGE.

MurF co-pelleted with MreB polymers but did not sediment or pellet on its own (Figure 31). To confirm the assay specificity, a control containing BSA and MreB showed that BSA remained in the supernatant and did not interact with MreB in a non-specific manner. Keeping a constant concentration of MreB (5 µM), the amount of MreB spun down in the pellet was increased with increasing the concentration of MurF (2.5 µM, 5 µM, 10 µM and 15 µM). Almost all MreB was polymerized and recovered in the pellet at a molar ratio of 1:3 (MreB:MurF). These results indicate that polymerization of MreB is favored in the presence of MurF.

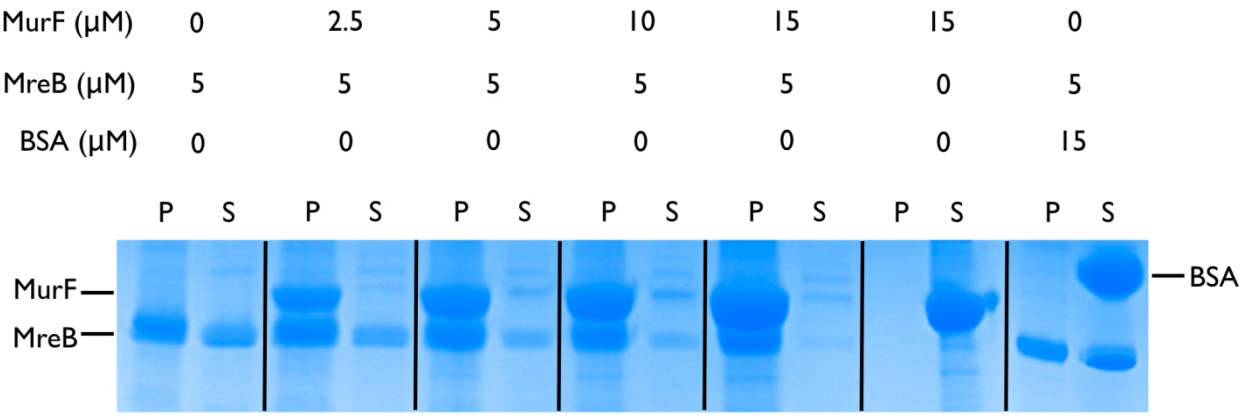

Figure 31: *In vitro* interaction of MreB and MurF from *C. pneumoniae* in co-pelleting assays.

MreB was polymerized in the absence and presence of increasing amounts of MurF. After centrifugation, equivalent volumes of supernatants (S) and pellets (P) were separated by SDS-PAGE

4.1.10.3 Studies on the *in vivo* interaction between chlamydial MreB, MraY and MurG proteins

During this study, a bacterial adenylate cyclase two-hybrid system (BACTH system, Euromedex) was used to analyze the interaction between MreB and the enzymes catalyzing the membrane bound steps of lipid II biosynthesis, MraY and MurG *in vivo*. In this assay, genes of interest were cloned into pUT18 and pKNT25 vectors in frame with gene fragments T18 and T25. The two recombinant plasmids were co-transformed into the adenylate cyclase deficient reporter strain *E. coli* BH101. Interaction between proteins fused to the two fragments (T18 and T25) mediates the reconstitution of active adenylate cyclase, which produces cyclic AMP (cAMP) and activates the *lac* and *mal* operon to produce β-galactosidase. A β-galactosidase assay was used to quantify the functional complementation of adenylate cyclase enzyme mediated by the interaction between two proteins.

Bacterial two-hybrid analyses showed that MreB is self-interacting confirming the results from the *in vitro* polymerization assays. Additionally, MreB showed interaction with both the integral protein MraY and the membrane associated cytosolic protein MurG. A relatively low β-galactosidase activity was expected from these assays because of the N-terminal localization of the adenylate cyclase fragments. In fusion with MraY, the adenylate cyclase fragment was located in the periplasm (predicted from topology studies [46] and using topology prediction program TMHMM [144]). Fused to MurG, the respective fragment was located closed to the membrane association site of the protein (predicted from crystal structure [145]).

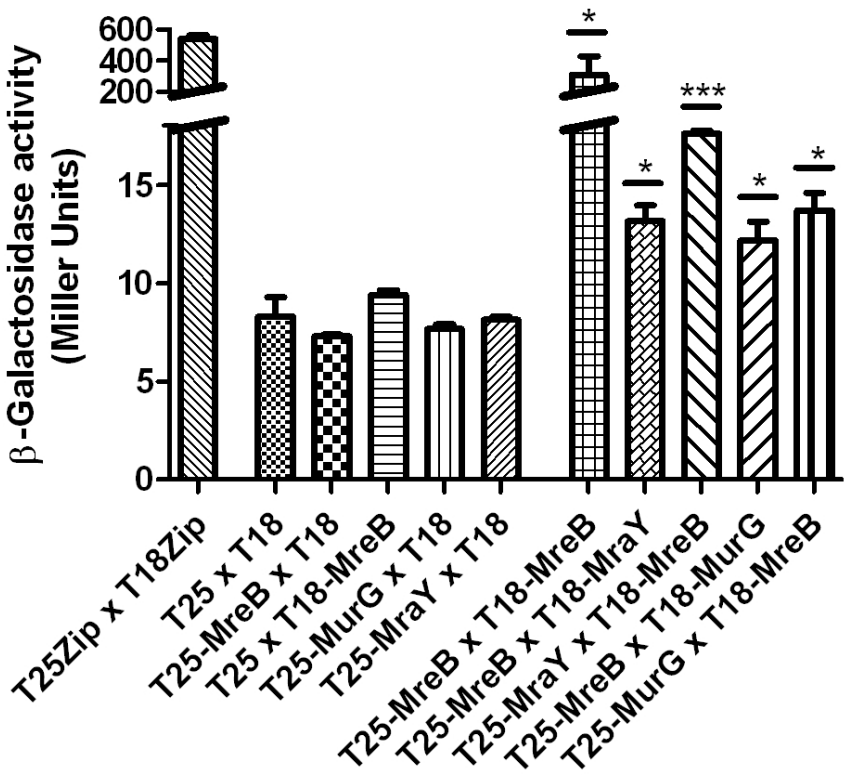

Figure 32: Bacterial two hybrid studies on *in vivo* interactions between chlamydial MreB, MraY and MurG.

Protein interactions were quantitatively determined by measuring β-galactosidase activities of three individually selected replicate co-transformants. The β-galactosidase activities are expressed in Miller Units (change in A_{420}/min/ml/OD_{600}) as mean values (± standard deviation). Unpaired t-test revealed statistical significance in comparison to empty vector negative control (T25×T18), *: two-tailed p-value≤0.05, *** two-tailed p-value≤0.001).

4.2 ANALYSIS OF CHLAMYDIAL GLYA RACEMASE ACTIVITY

The terminal dipeptide D-ala-D-ala of the pentapeptide side chain of lipid II is the target for the PBPs activity. Chlamydial genomes lack the *alr* gene encoding an alanine racemase that is responsible for D-alanine synthesis in other bacteria. It has been shown before that GlyA from *E. coli* can catalyze the interconversion between D- and L-alanine [98]. The racemase activity of chlamydial GlyA was investigated to check whether it could synthesize D-alanine in these endobacteria.

4.2.1 Cloning, over-production and purification of chlamydial GlyA

The pET-21b and pET-52b vectors were used to construct a GlyA with C-terminal His_6-tag and GlyA with both C-terminal His_6-tag and N-terminal *Strep*-tag respectively (Figure 33). Positive clones were selected by colony PCR and were confirmed by restriction reactions (Figure 34).

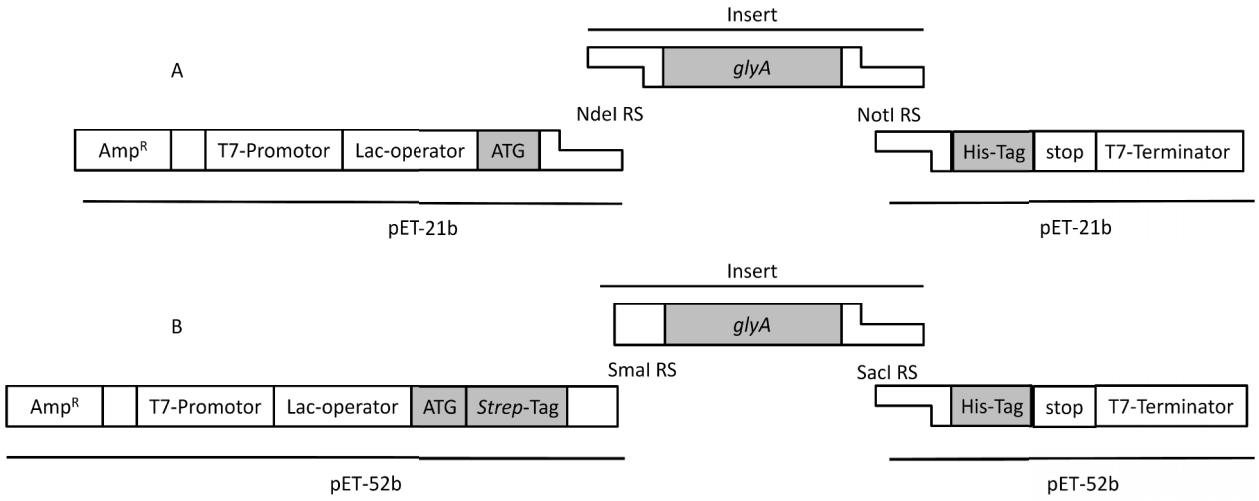

Figure 33: Schematic presentation of the recombinant plasmids pET-21b-*glyA* (A) and pET-52b-*glyA* (B).

RS = restriction site.

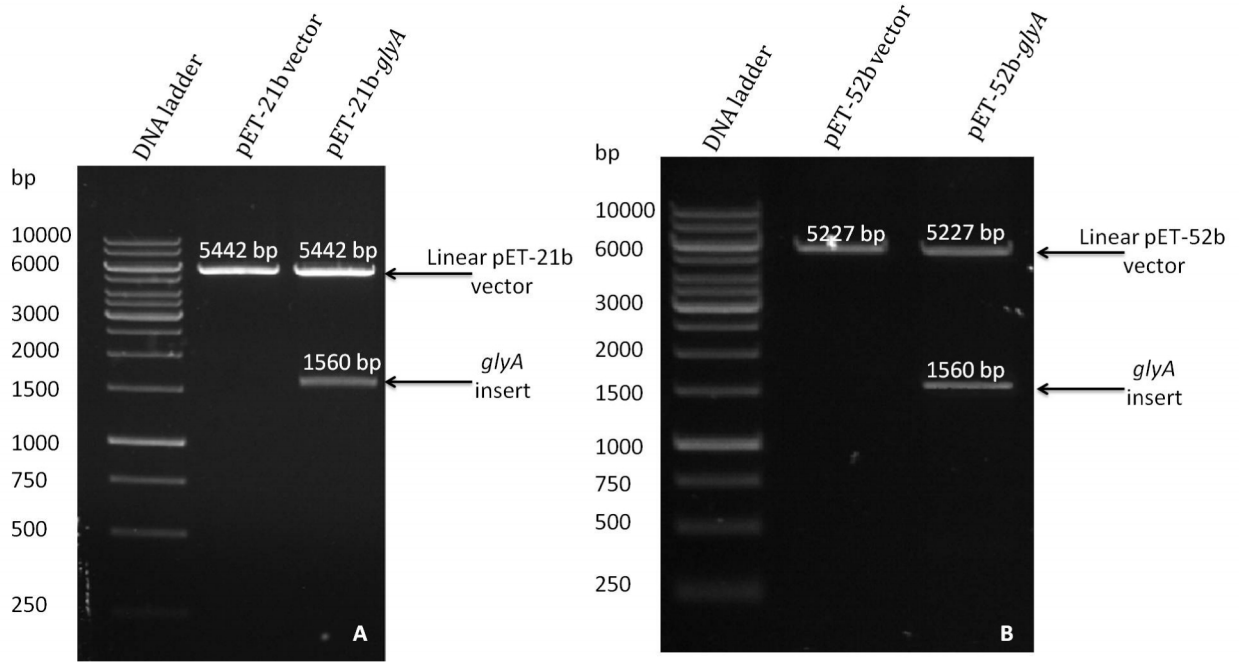

Figure 34: Detection of positive clones from recombinant plasmids pET-21b-*glyA* (A) and pET-52b-*glyA* (B) using restriction analysis and agarose gel electrophoresis.

One mg of plasmid DNA was restricted for 1 hour with NdeI and NotI (A) or SmaI and SacI (B). DNA ladder: 1 Kb DNA ladder (Fermentas)

4.2.1.1 Over-production and purification of recombinant GlyA

The His_6-tagged GlyA was purified from 1 liter culture after 4-5 hours of induction with 0.5 mM IPTG. The cleared lysate was prepared using a buffer containing 5 mM β-mercaptoethanol and 0.1 mM co-factor pyridoxal phosphate to increase the stability of GlyA. The lysis buffer contained also 25% glycerol to decrease the hydrophobic interactions between proteins in order to increase the protein purity. The cleared lysate was loaded on Ni-NTA resin columns, washed and then eluted with a buffer containing 250 mM imidazole (Figure 35). Because of the poor purity of GlyA-His_6 elution fractions, the double tagged *Strep*-GlyA-His_6 protein was prepared from the pET-52b-*glyA* plasmid. The double tag allowed purification on Ni-NTA followed by a second step using *Strep*-Tactin resin. The *Strep*-tag did not bind to the resin in the second purification and better purity was not achieved (data not shown). Gel filtration chromatography, an approach to separate proteins according to their sizes, was also tried to get better purity. GlyA was not recovered in the expected elution fractions, which might be a result of protein degradation (data not shown).

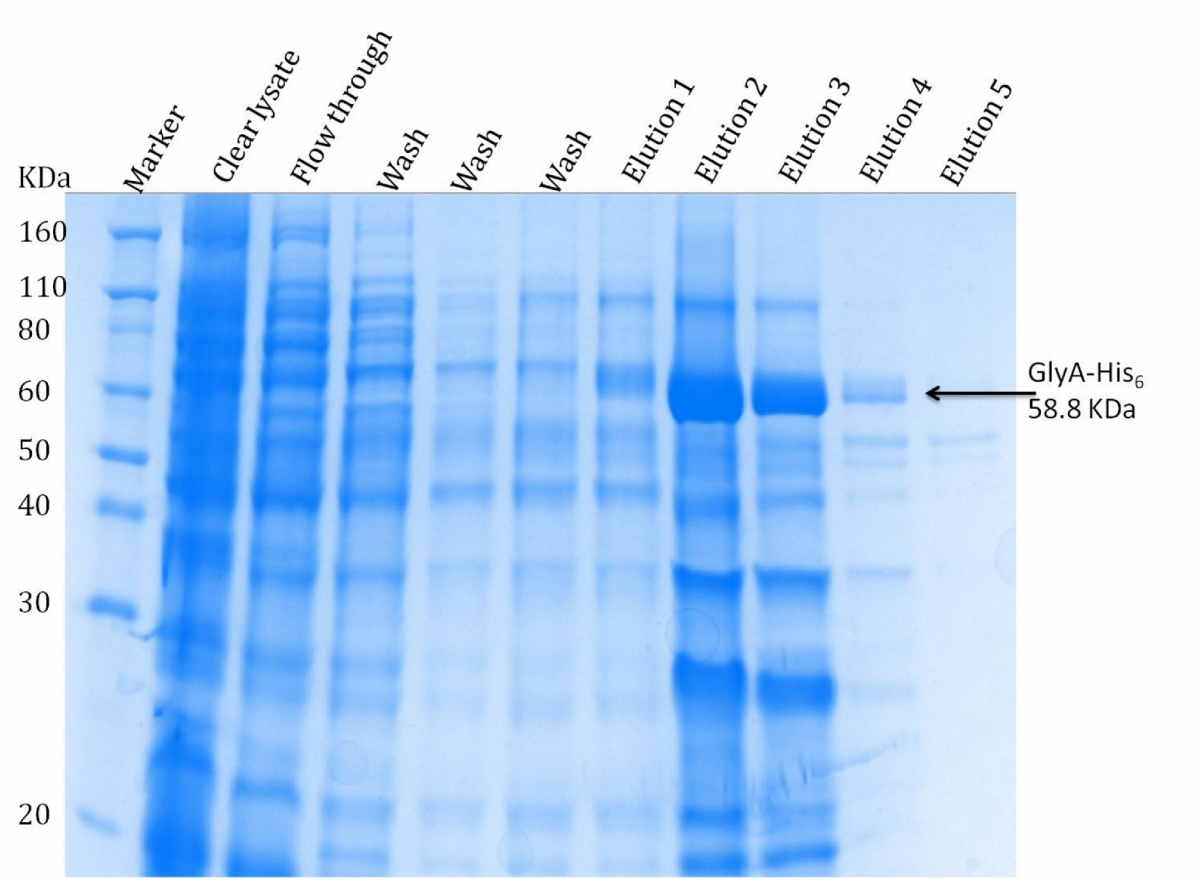

Figure 35: SDS-PAGE gel showing the protein purification of GlyA-His_6 (58.8 KDa) from *C. pneumoniae*.

Marker: Novex Sharp Pre-stained Protein Marker (Invitrogen)

4.2.2 Detection of racemase activity of chlamydial GlyA

The ability of the SHMT GlyA from *C. pneumoniae* to catalyze the interconversion between L-alanine and D-alanine was tested *in vitro*. GlyA was incubated with L- or D-alanine in presence of the co-factor pyridoxal phosphate. At the end of incubation time (4 or 24 hours), the amount of produced or consumed D-alanine were analyzed using a fluorescent coupled enzyme assay based on D-amino acid oxidase and horseradish peroxidase catalyzed reactions in the presence of the fluorescent substrate QuantaBlu.

The chlamydial GlyA catalyzed the conversion from L-alanine to D-alanine after 24 hours of incubation. Short incubation for 4 hours was not enough to detect the racemase activity *in vitro*, indicating weak catalytic activity of the chlamydial protein. GlyA from *C. pneumoniae* did not require the addition of external pyridoxal phosphate, during the

racemization reaction. This might indicate that pyridoxal phosphate bound to the enzyme during the purification process was enough or that chlamydial GlyA does not require pyridoxal phosphate for its activity. The physicochemical properties of several SHMTs from eukaryotic origin were investigated before and the SHMT from mung bean showed pyridoxal phosphate-independence [146] (Figure 36 A-B).

The enzyme bound pyridoxal phosphate has an absorption maximum in the range of 422-430 nm [99, 147, 148]. D-alanine was detected in this reaction using a fluorescent coupled-enzyme assay, in which fluorescence at 425 nm was measured. Removal of the enzyme bound pyridoxal phosphate before detecting D-alanine may be important to increase sensitivity of the assay.

On the other hand, conversion of D-alanine to L-alanine could not be detected after both 4 and 24 hours of incubation (Figure 36 C-D). This might indicate a weaker affinity towards D-alanine than L-alanine or low sensitivity of the applied test system to detect consumption of D-alanine in presence of high background of the D-amino acid. It was stated before that GlyA from *E. coli* binds L-alanine more tightly than D-alanine [98]. Establishing an assay to detect the release of L-alanine rather than consumption of D-alanine might help to increase the sensitivity to detect the racemase activity of D-alanine to L-alanine.

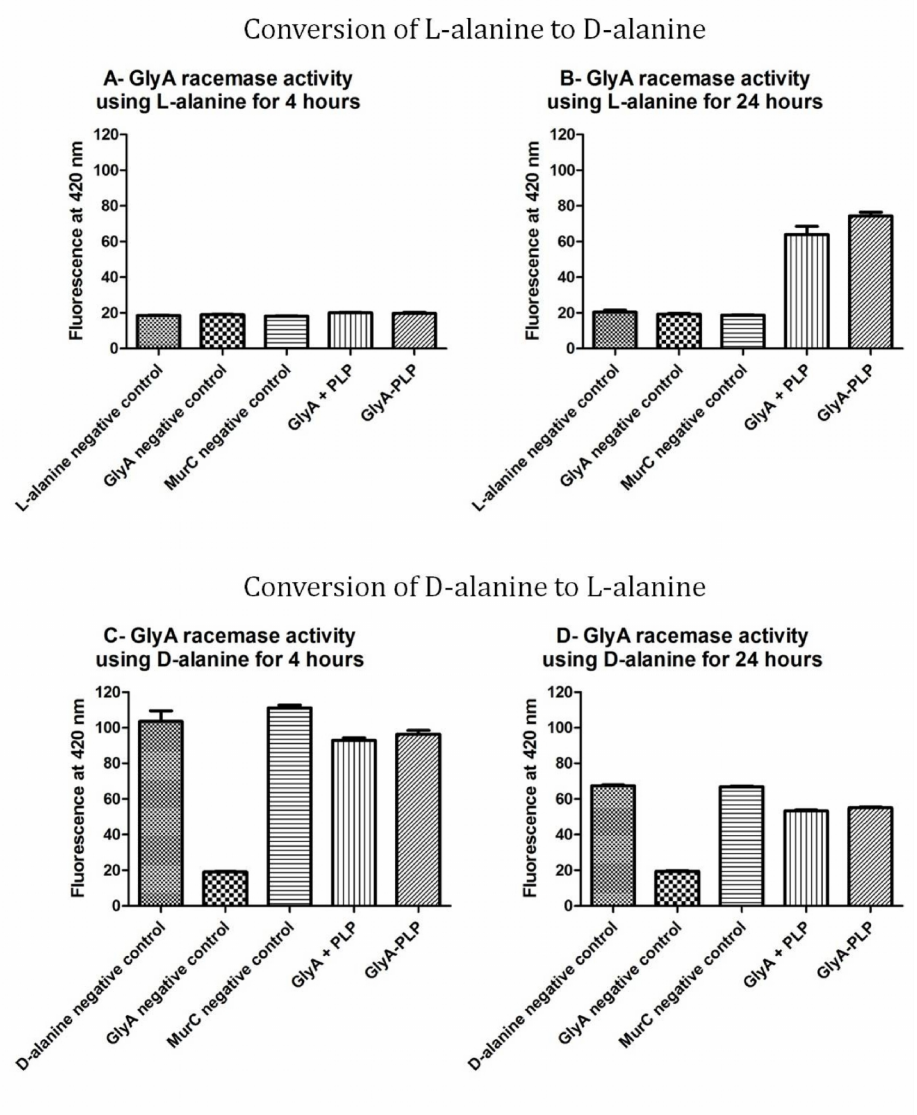

Figure 36: Racemase activity of chlamydial GlyA

GlyA was incubated with L- or D-alanine with or without the addition of pyridoxal phosphate (PLP). The D-alanine produced (A+B) or remaining (C+D) after 4 hours (A+C) or 24 hours (B+D) of incubation was detected with the activity of D-amino acid oxidase enzyme in presence of QuantaBlu fluorescent substrate.

To control for the background racemase activity of the high number of co-purified proteins, MurC-His_6 over-produced in the *E. coli* Bl21 strain and purified on Ni-NTA was used as a negative control with all reaction conditions and did not show any racemization activity. Because of different patterns of contaminating proteins in the elution fractions of the purified MurC-His_6 and GlyA-His_6, more controls are still required. Detection of racemase activity in harvested chlamydial RBs might be helpful to confirm the D-alanine biosynthesis in chlamydiae *ex vivo*.

4.3 FUNCTIONAL ANALYSIS OF CHLAMYDIAL PBPS

The sensitivity of chlamydiae to penicillin and other β-lactam antibiotics is attributed to the presence of three PBPs encoded in the chlamydial genome. Chlamydiae have three PBPs homologous to PBP2, 3 and 6a from *E. coli*. PBP2 and PBP3 are essential mono-functional transpeptidases in *E. coli*. On the other hand, the *E coli* PBP6a shows a weak *in vitro* carboxypeptidase activity. To get insights into the functionality of the chlamydial PBPs, cloning, over-production and purification of the three proteins are essential preparatory steps.

4.3.1 Cloning over-production and purification of chlamydial PBPs

Cloning, over-production and purification of PBPs, even by other species, are in general very challenging and difficult. It was supposed that secondary structures in the genes may hinder the cloning, and toxicity of the gene product may inhibit good levels of expression. The presence of membrane anchors may affect the feasibility of protein purification. For those reasons, a strategy using different promoter systems, purification tags and chaperones was applied. Chlamydial PBP2, PBP3 and PBP6a were cloned separately in pASK-IBA3, pASK-IBA5 and pET-21b to build the constructs shown in Figure 37, Figure 39 and Figure 41. Positive clones were selected by colony PCR and were confirmed by restriction endonuclease reactions (Figure 38, Figure 40 and Figure 42).

Protein expression in pASK-IBA vectors is under the control of *tetA* promoter/operator region. In absence of the inducer, anhydrotetracycline, the promoter is tightly repressed by constitutive expression of *tet* repressor gene encoded on the vector. Complete repression of the promoter was considered beneficial for the over-production of proteins with expected toxicity like PBPs. A disadvantage of the *tetA* promoter is its low strength (only 25% the strength of the T7 one). On the other hand, protein expression in pET-21b vector is under the control of the strong T7 promoter with a leaky constitutive expression, which might show problems with cloning and expression of genes encoding proteins with expected toxicity.

While pASK-IBA3 and pASK-IBA5 introduced a *Strep*-tag to the recombinant protein at its C-terminus and N-terminus respectively, pET-21b vector fused a His_6-tag to the C-terminus of the recombinant protein.

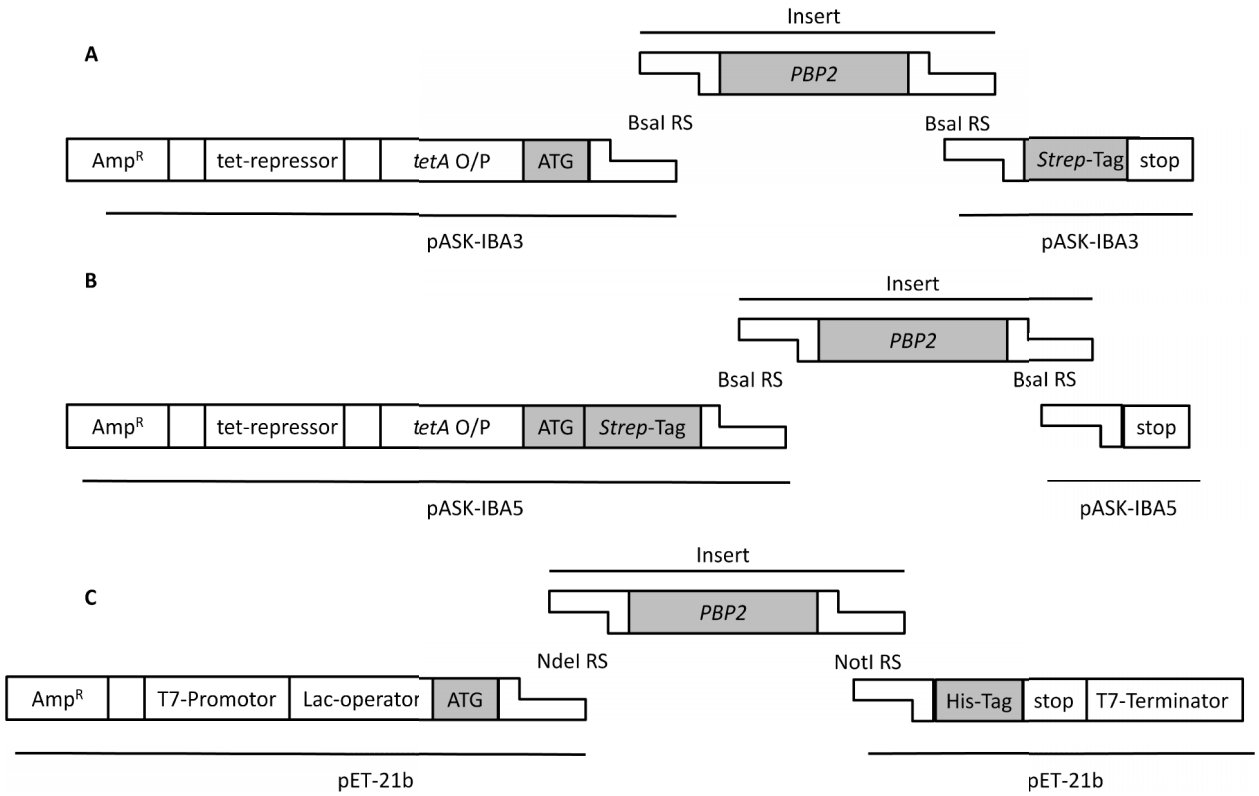

Figure 37: Schematic presentation of the recombinant plasmids pASK-IBA3-*PBP2* (A), pASK-IBA5-*PBP2* (B) and pET-21b-*PBP2* (C)

RS = restriction site

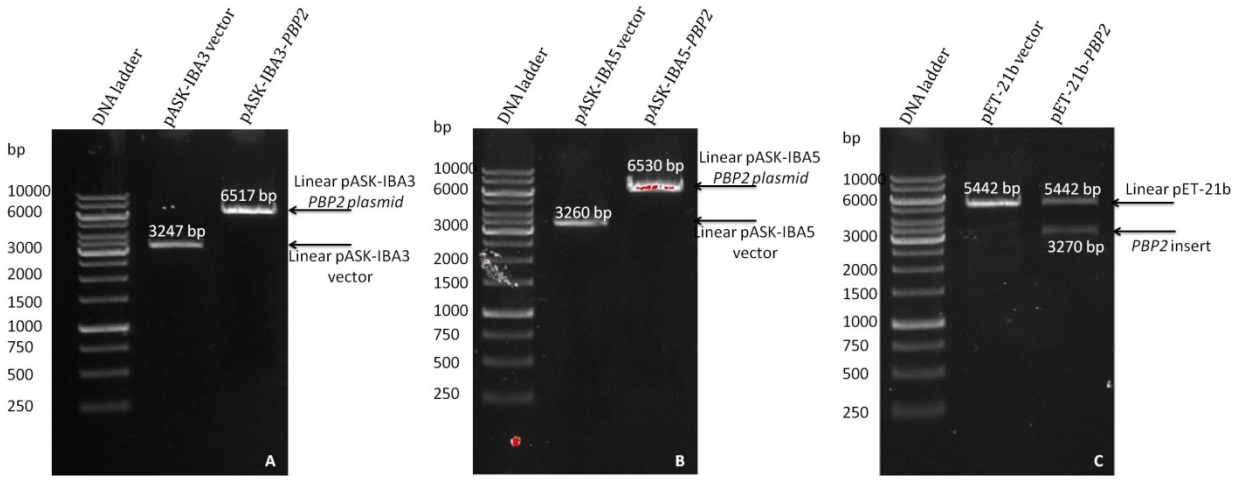

Figure 38: Detection of positive clones from recombinant plasmids pASK-IBA3-*PBP2* (A lane 3), pASK-IBA5-*PBP2* (B lane 3) and pET-21b-*PBP2* (C lane 3) using restriction analysis and agarose gel electrophoresis.

One mg of plasmid DNA was restricted for 1 hour with NdeI (A and B) or NdeI/NotI (C). DNA ladder: 1 Kb DNA ladder (Fermentas)

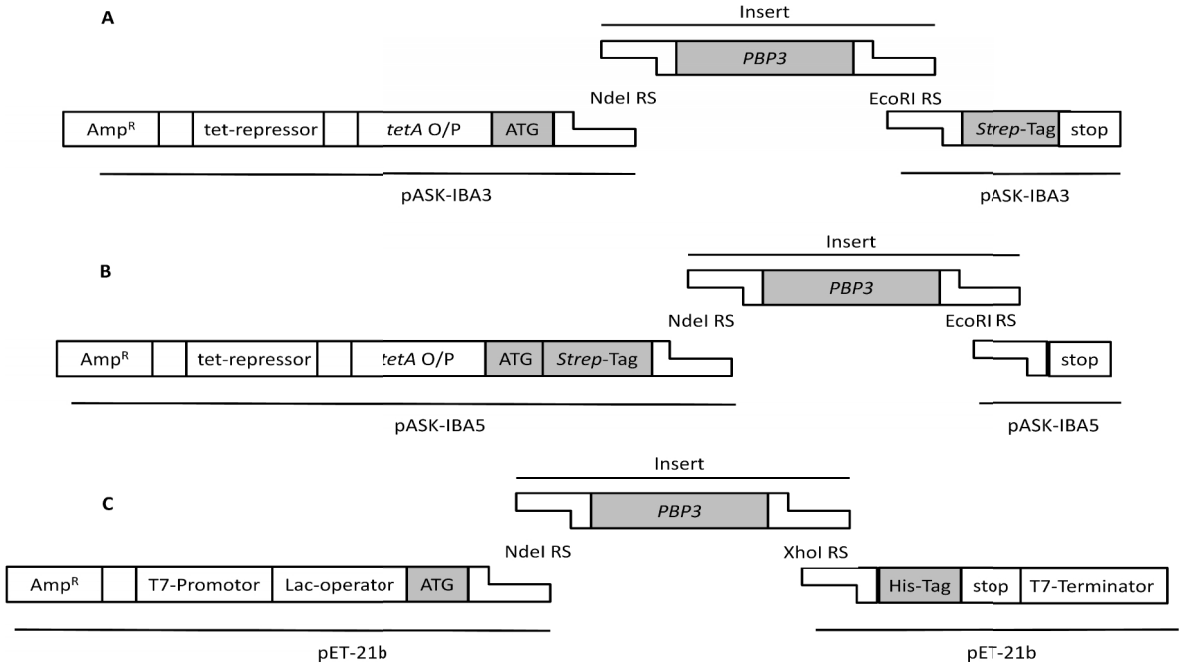

Figure 39: Schematic presentation of the recombinant plasmids pASK-IBA3-*PBP3* (A), pASK-IBA5-*PBP3* (B) and pET-21b-*PBP3* (C)

RS = restriction site

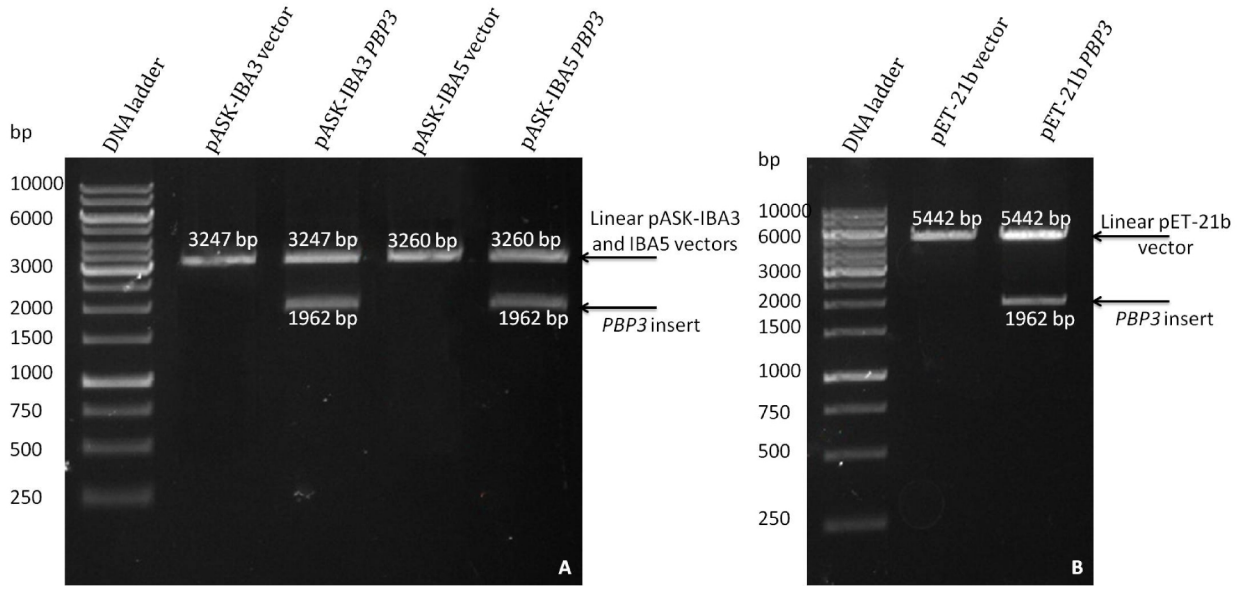

Figure 40: Detection of positive clones from recombinant plasmids pASK-IBA3-*PBP3* (A lane 3), pASK-IBA5-*PBP3* (B lane 5) and pET-21b-*PBP3* (C lane 3) using restriction analysis and agarose gel electrophoresis.

One mg of plasmid DNA was restricted for 1 hour with NdeI/EcoRI (A) or NdeI/XhoI (C). DNA ladder: 1 Kb DNA ladder (Fermentas)

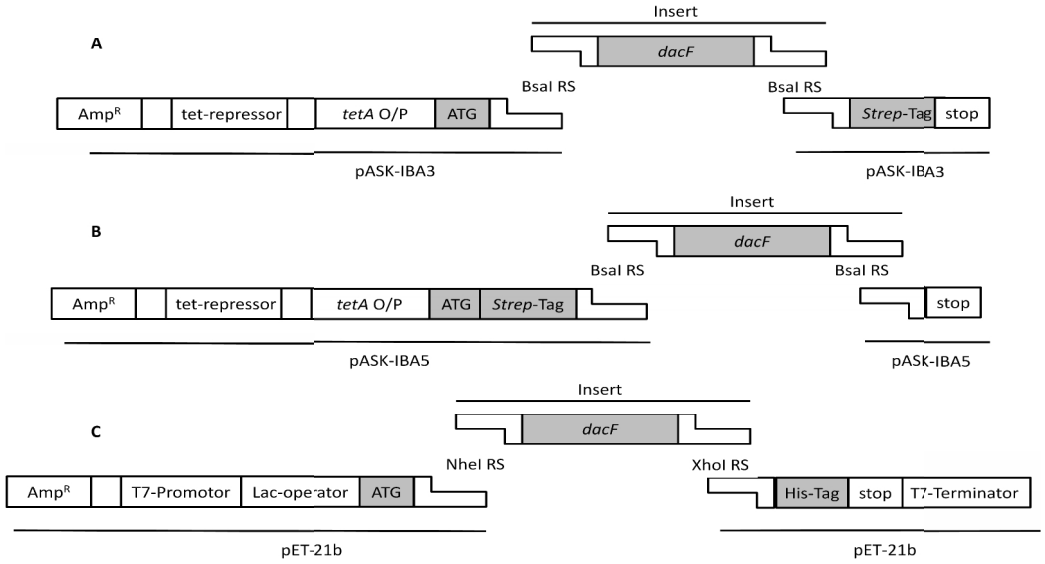

Figure 41: Schematic presentation of the recombinant plasmids pASK-IBA3-*dacF* (A), pASK-IBA5-*dacF* (B) and pET-21b-*dacF* (C)

RS = restriction site.

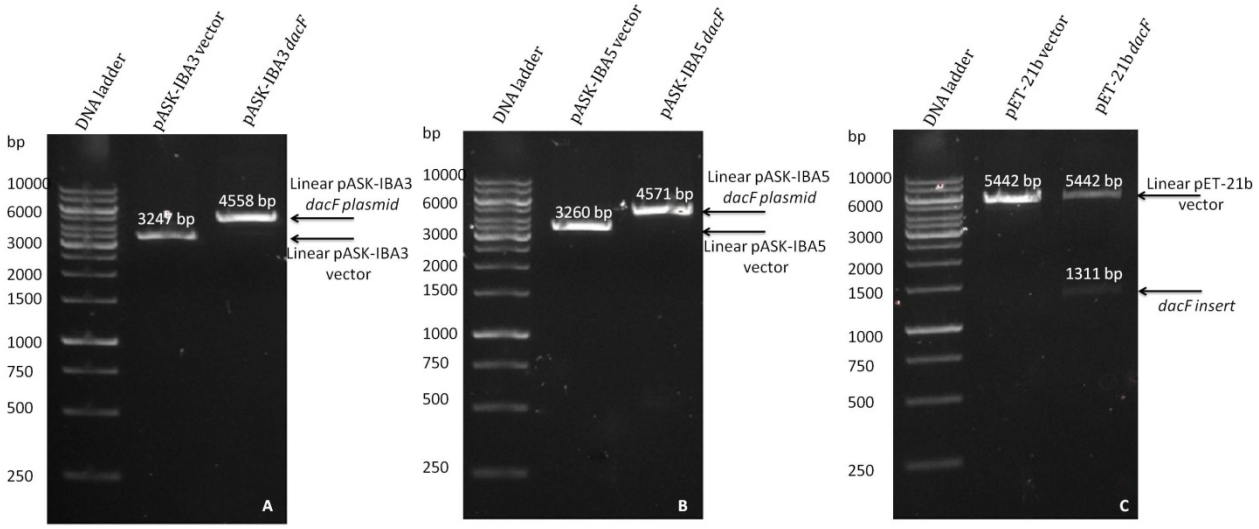

Figure 42: Detection of positive clones from recombinant plasmids pASK-IBA3-*dacF* (A lane 3), pASK-IBA5-*dacF* (B lane 3) and pET-21b-*dacF* (C lane 3) using restriction analysis and agarose gel electrophoresis.

One mg of plasmid DNA was restricted for 1 hour with XbaI (A and B) or NheI/XhoI (C). DNA ladder: 1 Kb DNA ladder (Fermentas)

4.3.2 Over-production and purification of recombinant chlamydial PBPs

Purification of *Strep*-tagged PBPs from the pASK-IBA3 recombinant plasmids was not achieved and attributed to one or more of the following reasons: (i) inaccessibility of the *Strep*-tag at the C-terminus, (ii) very weak or no protein expression because of its expected toxicity and/or (iii) formation of inclusion bodies that hinder the recovery of the protein in the soluble fraction during cleared lysate preparation. Moving the *Strep*-tag to the N-terminus using the pASK-IBA5 recombinant vectors disproved the first option concerning the accessibility of the tag. To check the protein expression and solubility, the soluble and insoluble fractions of the cleared lysates were tested by western blotting, followed by chemiluminescence detection of *Strep*-tagged proteins using *Strep*-Tactin horseradish peroxidase conjugate. A very weak signal was detected in the insoluble fraction in case of PBP3 cloned in pASK-IBA5 vector (data not shown). Despite the use of different detergents, PBP3 could not be solubilized and recovered from the insoluble fraction.

The T7 promoter system was used to overcome the weak expression level achieved with the pASK-IBA vectors. None of the three recombinant proteins showed sufficient over-expression from the pET-21b recombinant plasmids. This might be a result of the toxicity and/or misfolding of the PBPs.

Over-production and purification of the three chlamydial PBPs were successfully established exploiting T7 driven expression in combination with co-expression of chaperones and fusion of C-terminal His-tags.

To improve the expression of chlamydial PBPs, co-expression with pKJE7 (TaKaRa) chaperone plasmid encoding chaperones DnaK, DnaJ, and GrpE was used. Co-expression of a target protein with chaperones has been shown to increase recovery of expressed proteins in the soluble fraction. The induction of the three PBPs was greatly improved in presence of the chaperones but the proteins remained in the insoluble fraction of the cell lysate. Using N-lauroylsarcosine (0.5 %), all His_6-tagged PBPs could be solubilized and purified using Ni-NTA resin (Figure 43 and Figure 44).

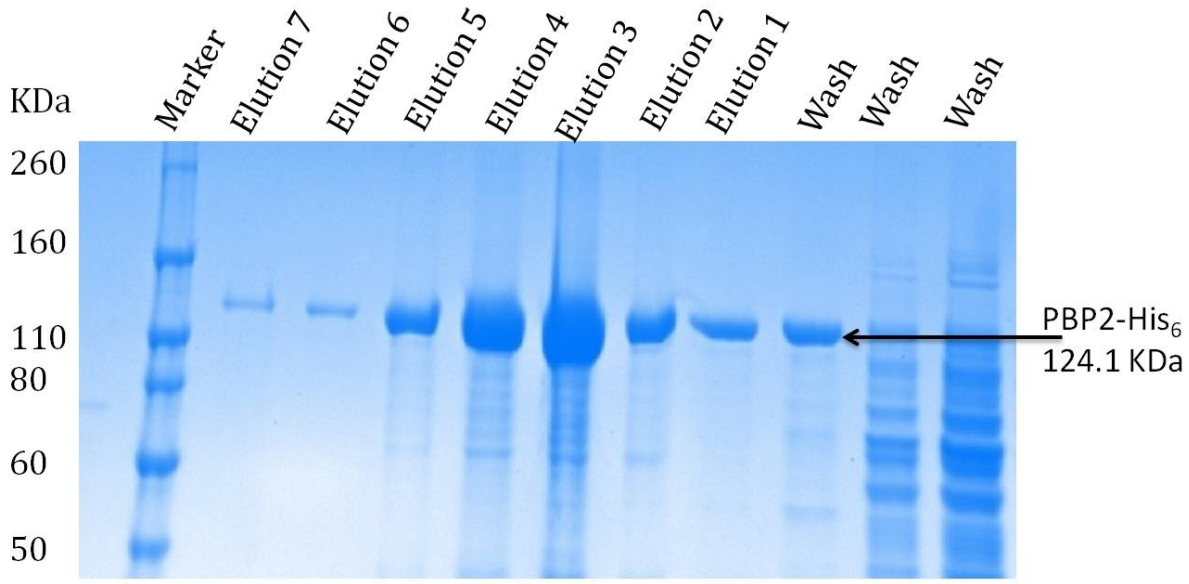

Figure 43: SDS-PAGE gel showing the protein purification of PBP2-His_6 from *C. pneumoniae*

Marker: Novex Sharp Pre-stained Protein Marker (Invitrogen)

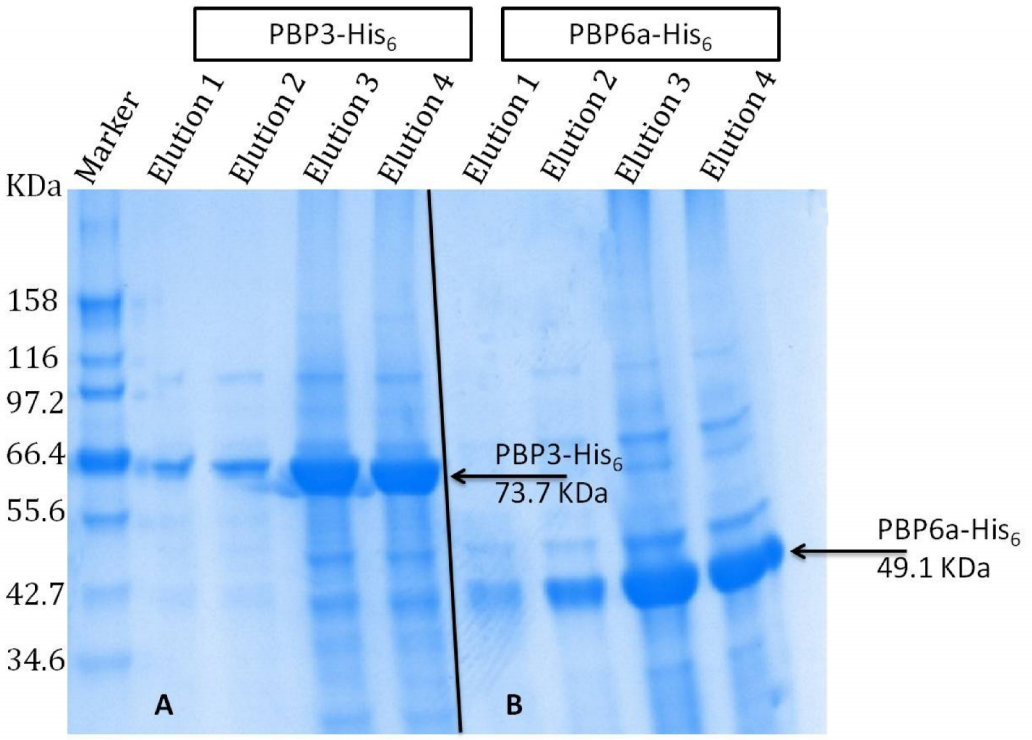

Figure 44: SDS-PAGE gel showing the protein purification of PBP3-His$_6$ (A) and PBP6a (B) from *C. pneumoniae.*

Marker: Broad Range protein marker (NEB)

The apparent molecular masses of the purified PBP3 (~66 KDa) and PBP6a (~43 KDa) as determined by SDS-PAGE were different from the calculated ones (73.3 and 49.1 KDa respectively). Discrepancies in the apparent molecular masses of chlamydial PBPs detected in membrane preparations of the RBs by binding to labeled penicillin were reported before. While Barbour *et al.* [120] showed that the apparent molecular masses of PBP2, PBP3 and PBP6a from *C. trachomatis* are 88, 61 and 36 KDa respectively, Stroy and Chopra [121] stated that the apparent molecular masses of these proteins are 110, 73 and 40 KDa respectively.

4.3.3 *In vitro* tests for transpeptidase activity of chlamydial PBP2 and PBP3

A recent study showed that the division of chlamydial RBs ceases by the second cycle of binary fission in the presence of penicillin [24]. Chlamydial PBPs might be responsible for processing of lipid II in the periplasm. Due to their sequence similarity to the transpeptidases PBP2 and PBP3 from *E. coli*, *in vitro* assays were first established to detect the functionality of the transpeptidase domain of the chlamydial proteins.

4.3.3.1 Using the natural substrates, lipid II and PG sacculi

In a recent study by Born *et al*. [106] the transpeptidase domain of a bi-functional PBP1A from *E. coli* was not able to exhibit a transpeptidase activity in the absence of a functional transglycosylase domain. Based on these results, mono-functional transpeptidases such as PBP2 and PBP3 from *C. pneumoniae* might not show *in vitro* activity disconnected from transglycosylation. That is why, a mutant of the bi-functional PBP2 from *S. aureus* (prepared by Anna Müller) with a functional transglycosylase domain and dysfunctional transpeptidase domain was included in an assay to detect the transpeptidase activity of the chlamydial PBP2 and PBP3. The principle of the reaction is based on analyzing the ability to attach lipid II to PG sacculi *in vitro*. PBPs were incubated with PG sacculi from *E. coli* W3110 or *B. subtilis* for 10 minutes before mixing with lipid II in presence or absence of 0.05% Triton X-100. The reaction was incubated at 30°C for 2 hours and centrifuged to remove the PG sacculi. The lipid content of the supernatant was extracted and analyzed on TLC to show whether lipid II was utilized as a substrate for transglycosylation and transpeptidation reactions.

Under the applied conditions, the PBP2 and PBP3 from *C. pneumoniae* did not exhibit a transpeptidase activity in absence or presence of the transglycosylase domain of PBP2 from *S. aureus*. Being from Gram-positive origin, the transglycosylase domain of the staphylococcal PBP2 mutant was not active when tested alone under the reaction conditions (pH 7.5) (Figure 45).

In a very recent study, Banzhaf *et al.* [149] showed that upon mixing the bi-functional *E. coli* PBP1A mutant, with a dysfunctional transpeptidase, and the mono-functional transpeptidase PBP2 from *E. coli*, no transpeptidase activity was detected *in vitro*. On the other hand, when the mono-functional PBP2 and bi-functional PBP1A wild-type proteins were combined in the assay, a significant increase in the overall transpeptidase activity was detected in comparison to reactions mediated with PBP1A alone. This increase was confirmed to be due to the transpeptidase activity of the mono-functional PBP2. These results indicate that establishing an assay using a bi-functional *E. coli* PBP, with functional transglycosylase and transpeptidase activities, together with the chlamydial PBP2 and PBP3 might be beneficial to detect putative transpeptidase activities *in vitro*.

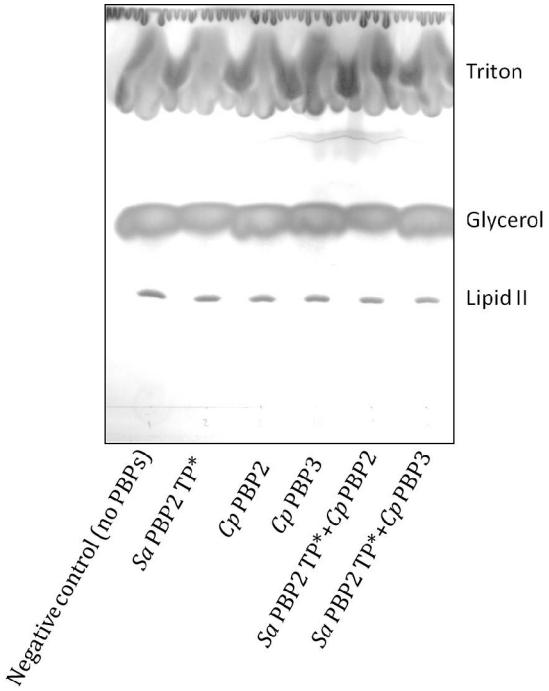

Figure 45: Investigation of transpeptidase activity of PBP2 and PBP3 from *C. pneumoniae*

PBPs (10-20 µg each) were mixed with PG sacculi on ice, then lipid II (2 nmol) was added. After 1 hour of incubation at 30 °C, the PG sacculi were removed by centrifugation. Supernatants were analyzed for changes in lipid II content using TLC. *Sa* PBP2-TP* is PBP2 from *S. aureus* with dysfunctional transpeptidase domain. *Cp: C. pneumoniae, Sa: S. aureus*

4.3.3.2 Using the artificial thioester S2d substrate

Another approach to detect the transpeptidase activity of the chlamydial PBP2 and PBP3 was detecting the ability of these proteins to hydrolyze the thioester N-benzoyl-D-alanylmercaptoacetic acid (S2d), an analogue of the bacterial cell wall stem peptide [135-137]. In this assay, the chlamydial PBP2 or PBP3 was incubated at 37°C with S2d in presence of 4,4'-dithiopyridine, which has been shown to couple with the S2d hydrolysis product and to increase the sensitivity of the assay [135]. The increase of the absorbance at 325 nm was used as a measure for the transpeptidase activity.

First trials did not show differences between the samples containing chlamydial proteins and a negative control in which the PBPs were replaced by Tris buffer (Figure 46). The detected increase in the absorbance at 325 nm is attributed to the auto-hydrolysis of the thioester.

Under applied conditions, chlamydial PBP2 and PBP3 did not exhibit transpeptidase activity in both test systems using natural and artificial substrates. More conditions should be tested to verify these results in both test systems.

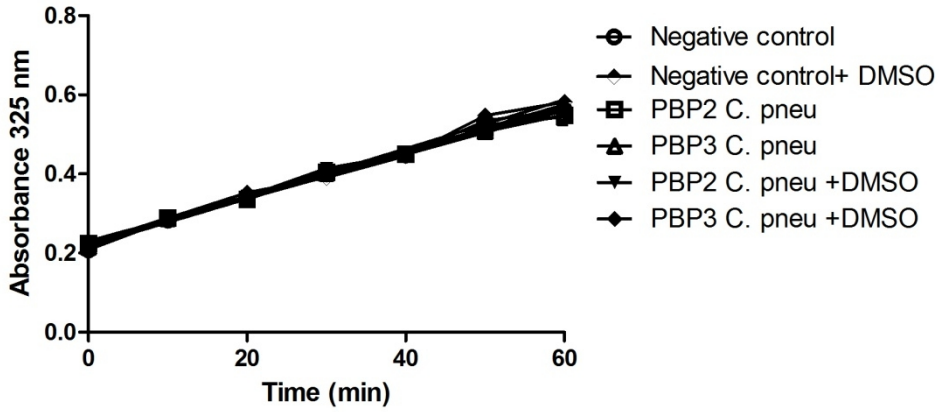

Figure 46: Investigation of transpeptidase activity of chlamydial PBP2 or PBP3 using the thioester S2d as a substrate.

PBP2 or PBP3 from *C. pneumoniae* was incubated with 1 mM S2d and 0.8 mM 4,4'-dithiopyridine in a buffer system containing 10 mM phosphate buffer pH 7 with or without 10% DMSO. Absorbance at 325 nm was measured every 10 minutes for 1 hour. A negative control using Tris buffer instead of PBPs was performed as well.

4.3.4 *In vitro* tests for carboxypeptidase activity of chlamydial PBP6a

Due to sequence similarity to the carboxypeptidase PBP6a from *E. coli*, test systems were first established to detect the functionality of the carboxypeptidase domain of the chlamydial protein. To examine whether the chlamydial PBP6a has a carboxypeptidase activity, the enzyme was tested for the release of the terminal D-alanine from the natural pentapeptide substrate lipid II or the artificial tripeptide substrate Nα,Nε-diacetyl-L-lys-D-ala-D-ala. Incubation of lipid II with a carboxypeptidase leads to the hydrolysis of the terminal D-alanine and the production of lipid II variant with tetrapeptide stem, which is expected to migrate differently from lipid II containing pentapeptide stem on TLC. For the artificial substrate, the released D-alanine was detected using the fluorescent-coupled enzyme assay described in section 4.2.2

In both test systems, no carboxypeptidase activity was detected for PBP6a from *C. pneumoniae* (Figure 47). This might be due to very weak activity of the chlamydial protein that has been shown for several *E. coli* carboxypeptidases as well. In a recent study using soluble variants *E. coli* PBP6, the protein showed very weak activity *in vitro* (one seventh the activity of PBP5, the major carboxypeptidase in *E. coli*) using the artificial substrate Nα,Nε-diacetyl-L-lys-D-ala-D-ala. On the other hand, the *E. coli* protein showed no carboxypeptidase activity with the pentapeptide natural substrate [150]. The PBP6b, another carboxypeptidase in *E. coli* encoded by *dacD* gene showed very weak activity in an *in vivo* study as well [151]. Based on these results, very weak enzymatic activity is highly expected for the chlamydial PBP6a.

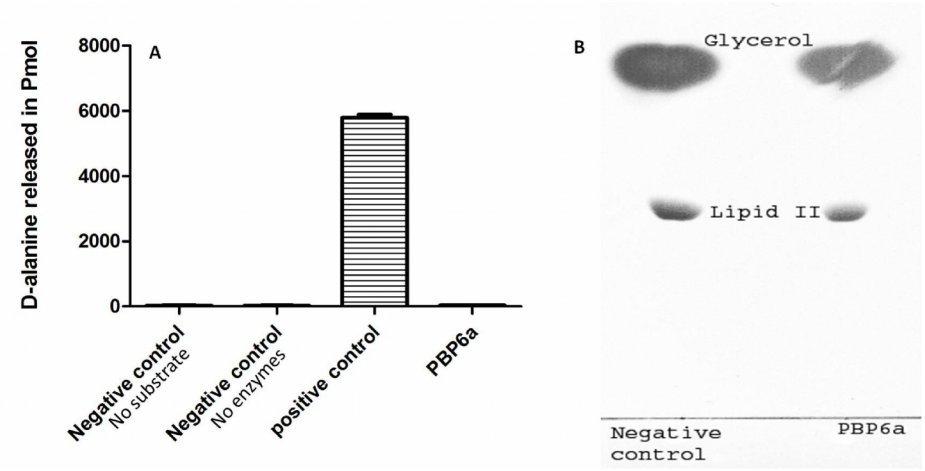

Figure 47: Investigation of carboxypeptidase activity of PBP6a from *C. pneumoniae* using the artificial substrate Nα,Nε-diacetyl-L-lys-D-ala-D-ala (A) and the natural pentapeptide substrate lipid II (B)

(A) D-alanine released was detected with the activity of D-amino acid oxidase in the presence of fluorescent substrate QuantaBlu. For the positive control a carboxypeptidase form *E. coli* was used. (B) After incubation with PBP6a, no change in the lipid II migration was detected.

4.3.5 *In vivo* interaction between chlamydial PBP3 and AmiA

In *E. coli*, the amidase AmiA cleaves the amide bond between MurNAc and the first amino acid in the peptide side chain L-alanine [152]. It is thought that chlamydial AmiA and PBP3 may play an important role in the processing of lipid II and the recycling of the lipid carrier C_{55}-P. Bacterial two hybrid analyses (done by bachelor student Karolina Schulz) suggested that chlamydial PBP3 interacts with AmiA (Figure 48). A relatively low β-galactosidase activity was expected from assays that take place in the periplasm.

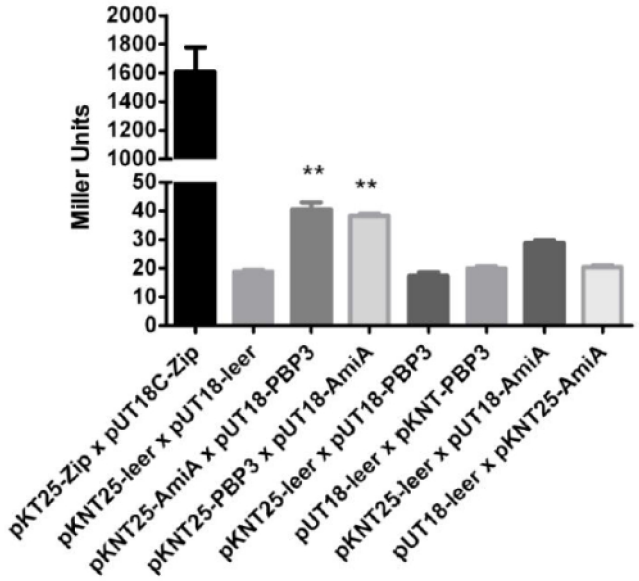

Figure 48: Bacterial two hybrid studies on *in vivo* interactions between chlamydial PBP3 and AmiA (K. Schulz)

Protein interactions were quantitatively determined by measuring β-galactosidase activities of three individually selected replicate cotransformants. The β-galactosidase activities are expressed in Miller Units (change in A_{420}/min/ml/$OD6_{00}$) as mean values (± standard deviation). Unpaired t-test revealed statistical significance in comparison to empty vector negative control (T25×T18), **: p-value≤0.01

5 DISCUSSION

Compared to free living bacteria like *E. coli*, prokaryotic intracellular pathogens have a remarkabley reduced genome size. *C. trachomatis* [25], *Mycoplasma pneumonaie* [153], *Rekettsia conorii* [154] and *Rekettsia prowazekii* [154, 155] have genome sizes ranging between 0.9-1.2 Mb, while *E. coli* [156] has a genome size of about 4.6 Mb. The reduced genome is suggested to be a result of hindered uptake of foreign DNA in these organisms because of their intracellular environment [157]. Other reasons include differential loss of genes that lost their functions or genome decay with increased adaptation to host cells [158]. For example, many genes coding for proteins involved in amino acid biosynthesis and some regulatory proteins have been discarded from the genomes of *R. prowazekii* and *M. genitalium* [159, 160]. Genome of *C. trachomatis* lacks the gene encoding thymidylate synthase required for the synthesis of deoxythymidine monophosphate [25] supporting the evidence that the latter is acquired from the host cell [161, 162].

During adaptation to the isotonic intracellular environment, chlamydiae did not need to keep a functional cell wall to guard against osmotic rupture and a cell wall has never been reliably detected. Surprisingly, chlamydiae retained the biosynthetic pathway of cell wall precursor lipid II [2, 25]. Additionally, chlamydiae lack the essential organizer of cell division FtsZ but harbor the cytoskeletal protein MreB [25]. These findings opened a discussion on how chlamydiae can organize its cell division in absence of the cell division driving FtsZ and the role of the shape-maintaining cytoskeletal protein MreB despite of their spherical shape.

Cell division and cell wall biosynthesis in prokaryotes are driven by partially overlapping multiprotein machineries. Absence of tight coordination between the activities of these machineries may disrupt cell integrity. Recently, it was proposed that conservation of lipid II biosynthesis in cell wall-lacking endobacteria may reflect the intimate molecular linkage of cell wall biosynthesis and cell division and thus a vital role of the bactoprenol bound precursor in cell division [95].

In this study, it was investigated whether MreB plays a role in spatially organizing lipid II biosynthesis reactions in chlamydiae. The functionality of MreB was investigated. Results from *in vitro* light scattering and sedimentation assays showed that chlamydial MreB polymerized. The *in vitro* results were supported by BACTH analysis, in which MreB showed a significant self-interaction *in vivo*. Polymerization of chlamydial MreB was favored in slightly acidic buffers (pH 6.5), in presence of Mg^{+2} and in presence of MurF. The polymerization was strongly inhibited at K^+ concentration as low as 20 mM, which was shown recently for MreB from *B. subtilis* [140]. *In vivo* studies proved that *B. subtilis* MreB can polymerize under physiological conditions (e.g. [163, 164]) with K^+ concentrations of about 300 mM [165]. Modulation and control of MreB polymerization *in vivo* may be affected by the interplay of enhancing mechanisms (protein interaction) and inhibitory mechanisms (salt susceptibility).

MreB from *C. pneumoniae, B. subtilis* and *T. maritima* show more that 54% amino acid identities. Despite these high sequence similarities, the three proteins differ in their dependence on nucleotides for polymerization and their susceptibility towards A22. Like all actin homologs, chlamydial MreB is a functional ATPase, nevertheless, in contrast to MreB from *T. maritima* [130], the proteins from *C. pneumoniae* and *B. subtilis* [140] polymerize independently from ATP.

A22 is frequently used *in vivo* studies on the bacterial MreB cytoskeleton. *E. coli* loses its rod shape upon treatment with A22 [141]. This compound was identified *in vitro* as a competitive inhibitor of ATP binding to MreB from *T. maritima* and A22-bound MreB has a greatly reduced affinity for polymerization [143]. The minimal inhibitory concentration (MIC) of A22 towards *B. subtilis* is extremely high (100 mg/L) comparable to MreB-lacking spherical bacteria *S. aureus* (>100 mg/L) and in contrast to susceptible organisms like *E. coli* and *Salmonella typhimurium* with MIC of 3.13 mg/L [66]. In accordance with ATP-independent polymerization, A22 did not inhibit polymerization of wild-type and C345A mutant MreB *in vitro*. Moreover, A22 did not inhibit ATP hydrolysis by chlamydial MreB indicating that the compound cannot bind the nucleotide-binding pocket of MreB from *C. pneumoniae*.

A22 affects bacterial growth not only by acting on MreB but also on (an)other essential unknown target(s). This has been shown in several recent reports on (i) *E. coli* mutant lacking MreB, (ii) an A22 resistant *Anabaena* mutant strain retaining a wild-type *mreB* gene, and (iii) rod-shaped bacteria containing (*C. crescentus*) or not containing (*Sinorhizobium meliloti, Agrobacterium tumefaciens*) MreB [166, 167]. Further studies dealing with the *in vivo* effect of A22 on chlamydiae, bacteria with reduced genome size and A22-resistant MreB, might be of importance to identify these unknown targets.

In this project, it was shown that chlamydial MreB interacts with MurF, MraY and MurG, three key components of lipid II biosynthesis. MreB and MurG have been shown to localize at the division site in *E. coli* and *C. crescentus* [70, 168, 169]. Moreover, in a very recent study [170], BACTH system analyses showed that chlamydial MreB interacts with the multifunctional cell division protein FtsK.

From the last discussed results and based on the hypothesis that lipid II conservation in cell wall-less bacteria is required for functional cell division [95], MreB could be involved in maintaining coordinated cell division via tethering the biosynthesis of lipid II to the chlamydial septum (Figure 50).

This hypothesis regarding chlamydial MreB is supported by a recent study on L-forms of *B. subtilis* [171]. L-forms are atypical bacteria that are cell wall-deficient and able to grow as spheroplasts [172]. They are osmotically fragile and seem to lack the usual coordinated binary fission process of division of typical bacteria [173]. The stable L-forms generated in the study by Leaver *et al*. did not require FtsZ for cell division [171]. It was postulated a FtsZ independent extrusion-resolution mechanism in which a cytoskeletal system (built up by *B. subtilis* actin homologs MreB, Mb1 or MreBH) or an active chromosome segregation system might provide force generation. Moreover, L-form mutants lacking D-alanine racemase, responsible for D-alanine synthesis from L-alanine, can grow in the absence of D-alanine supplements, and electron microscopy did not detect any residual cell wall outside the boundary of the cytoplasm or at the division site. These findings are in good agreement with the hypothesis that monomeric cell-wall precursor lipid II is required for cell division in *Chlamydiaceae* as bacteria of these family lack a cell wall and lack also D-alanine racemase [95].

During longitudinal growth of rod-shaped bacterial like *E. coli,* the membrane spanning proteins, MreC and MreD, play an important role in connecting the cytoplasmic MreB-organized-precursor-biosynthesis machinery to the PBP2-catalyzed incorporation of lipid II in the periplasm (Figure 6A) [65, 70]. While MreC and MreD are encoded in the genomes of elongated bacteria in one operon together with MreB [65], both proteins are missing in chlamydiae. For spherical bacteria, shape maintaining functions and spacial organizing of PBP2 reactions are dispensable. Therefore, chlamydiae may have not retained MreC and MreD.

Under stress conditions, chlamydiae can enter a reversible non-infectious but viable state known as persistence. Treatment of chlamydiae-infected cells with the cell wall biosynthesis inhibitor penicillin can induce such a state of persistence, in which cell division is arrested by the second cycle. RBs continue to grow and synthesize chromosomal and plasmid DNA forming enlarged and aberrant RBs known as penicillin bodies (Figure 3). Upon removal of penicillin, a normal developmental cycle is recovered [24]. Binding of penicillin to chlamydial PBPs may interrupt processing of lipid II and cell division might be arrested due to depletion of the bactoprenol carrier. Sensitivity of chlamydiae to treatment with cell wall synthesis inhibitors like penicillin, D-cycloserine and bacitracin in absence of clear-cut biochemical evidence for the presence of PG cell wall is a paradox know as chlamydial anomaly.

Besides the conservation of lipid II biosynthesis pathway in the chlamydial genome, other findings confirm the functional biosynthesis of cell wall precursors in chlamydiae. The nucleotide oligomerization domain (Nod) proteins, interacellular receptors recognizing different fragments of PG [85, 87-89], are indispensible for sensing and generating immune response to intracellular infections with *C. pneumoniae* [83], and Nod1/Nod2 knockout mice showed delayed innate immune response and bacterial clearance [82].

Chlamydial genomes encode 3 PBPs, which are the target for the action of penicillin [120]. D-cycloserine inhibits the activity of both alanine racemase (Alr) and D-alanyl-D-alanine ligase (Ddl) [174]. No clear evidence until now confirmed that chlamydial genomes encode any homologs of Alr but Ddl is encoded as a fusion protein with MurC [25, 175,

176]. Sensitivity of chlamydiae to bacitracin shows that recycling of the lipid carrier bactoprenol is essential for chlamydiae and its developmental cycle. Bacitracin binds bactoprenol pyrophosphate (C_{55}-PP) inhibiting its dephosphorylation and recycling. Interestingly, chlamydiae are resistant to fosfomycin [93], a bacterial cell wall synthesis inhibitor that binds irreversibly to and inhibits the activity of MurA due to its structural similarity to phosphoenolpyruvate (PEP) [177]. Sequence analysis of MurA from *C. trachomatis* showed a cysteine to aspartate change in a key residue within the active site of the enzyme [93]. The same amino acid change was previously described in MurA mutant from *E. coli* to induce resistance to fosfomycin [178]. Additionally, the same natural amino acid change was described in *Mycobacterium tuberculosis*. Changing this amino acid back from aspartate to cysteine renders MurA from *M. tuberculosis* sensitive to fosfomycin [179].

Although the chlamydial anomaly is known since the early 90s, the molecular basis of this phenomenon is not elucidated yet. Until today, the activity of cytosolic chlamydial proteins MurA [93], MurC/Ddl [90, 94], MurE [91] and MurF [92], transmembrane protein MraY [95] and the membrane associated protein MurG [95], involved in lipid II biosynthetic pathway have been proven. Moreover, an alternative pathway was detected for the biosynthesis of the third amino acid in the peptide side chain, m-DAP [96]. Despite all these findings supporting the idea that chlamydiae synthesize lipid II, the exact structure of lipid II-peptide side chain in chlamydiae is not yet confirmed.

MurC, the enzyme responsible for the addition of the first amino acid in the stem peptide, from *C. trachomatis* uses L-alanine, L-serine and glycine as a substrate *in vitro* with comparable efficiency [90]. Indeed, this lack of specificity *in vitro* makes the amino acid in the first position of the stem peptide not clear and depends mainly on the availability of the amino acids in the intracellular pool [90].

Chlamydiae do not encode glutamate racemase (MurI), the enzyme needed for the synthesize of D-glutamate from its L-isomer [2]. Additionally, the functionality of the chlamydial MurD, the ligase enzyme responsible for the addition of the second amino acid (D-glutamic acid) to the peptide side chain, is not proven until now. Despite the absence of MurI and doubts about the MurD activity, MurE from *C. trachomatis* can recognize MurNAc-L-alanine-D-glutamate *in vitro* [91].

For the biosynthesis of the third amino acid in the peptide stem, m-DAP, an aminotransferase pathway was described in chlamydiae using the activity of CT390 and DapF proteins [96]. The addition of the third amino acid is catalyzed by the activity of MurE ligase. Patin *et al.* showed that chlamydial MurE ligase is functional and can complement the activity in *E. coli* mutant with impaired MurE [91]. *In vitro*, chlamydial MurE recognized not only m-DAP as a substrate, but meso-lanthionine and D-lysine as well but with much weaker activity [91].

In the last cytoplasmic step of lipid II biosynthesis, MurF ligase adds the dipeptide D-ala-D-ala to the peptide stem completing the pentapeptide side chain. In a recent study, chlamydial MurF could complement the growth of *E. coli murF* temperature-sensitive lysis mutant at its restrictive temperature [92]. The chlamydial enzyme prefers m-DAP at the third position of the peptide stem to L-lysine, the latter is found mainly in Gram-positive bacteria [92]. Moreover, the enzyme can catalyze the addition of the dipeptide D-ala-D-ser and D-ala-gly as efficient as D-ala-D-ala [92]. The capability of chlamydiae to synthesize the dipeptide D-ala-D-ala via the activity of the Ddl domain of the MurC-Ddl fusion protein was confirmed *in vitro* [94].

Although the results on functionality of chlamydial MurF and Ddl domain of the fusion protein MurC-Ddl and sensitivity of chlamydiae to D-cycloserine and penicillin suggest that D-alanine is present in chlamydial lipid II, the source of this D-amino acid in chlamydiae is still unknown. Chlamydiae lack the alanine racemase Alr, which is responsible for D-alanine synthesize in other bacteria. Moreover, genes encoding homologs to D-amino acid transaminases, enzymes that can synthesize D-amino acids, could not be detected in chlamydial genomes [2]. Additionally, no evidence supports the hypothesis that D-alanine is supplied to chlamydiae from the mammalian hosts as they do not contain sufficient concentrations of D-amino acids [180]. *alr* knock-out mutants of *Listeria monocytogenes* could not survive within mammalian cells without exogenous supplement of D-alanine [181]. Together, these findings support the hypothesis that chlamydiae synthesize D-alanine on their own.

One aim of this project was to show how chlamydiae could synthesize D-alanine. Chlamydial genome encodes a serine hydroxymethyl transferase (SHMT) GlyA. The *glyA*

gene was initially transferred from an actinobacteria to a common ancestor of the family Chlamydiales by lateral gene transfer [101]. The hypothesis that chlamydial GlyA might serve as a source for D-alanine required for lipid II synthesis in such bacteria is supported by the results of a recent study on an *alr/dadX*-deficient *E. coli* strain. This strain exhibited a D-alanine auxotrophic phenotype. Transcriptome analysis of D-alanine prototrophic revertants showed a significant upregulation of genes involved in methionine synthesis including GlyA [100]

In this study, the preliminary results on the *in vitro* racemase activity of the chlamydial SHMT GlyA show that the protein may provide a way to synthesize D-alanine by these bacteria. Chlamydial GlyA could catalyze the conversion of L-alanine to D-alanine, which was not true for the negative controls without GlyA or with MurC. These findings provide another indication that lipid II in chlamydiae contains the terminal dipeptide D-ala-D-ala. Lipid II and nascent chains of PG containing D-ala-D-ala are the substrate for the reactions catalyzed by PBPs.

A complete cycle of lipid II biosynthesis and processing, including translocation across the membrane and recycling of the bactoprenol carrier is needed to maintain a coordinated function of the divisome machinery. Until now, the fate of lipid II in chlamydiae is unclear. While these obligate intracellular pathogens encode the flippase FtsW, an integral membrane protein, which translocates lipid II to the outer side of the cell membrane [25, 49], their genomes lack other enzymes essential for the processing of lipid II in the periplasm. Bacteria of the family *Chlamydiaceae* lack transglycosylases and endopeptidases that are needed to link the sugar units of lipid II to form glycan chains and to cleave peptide bridges between cross-linked glycan strains, respectively [25]. Additionally, they do not encode pyrophosphorylases, which catalyze in *E. coli* the recycling of the lipid carrier C_{55}-P by dephosphorylating its pyrophosphate form C_{55}-PP [52-54]. Moreover, chlamydial genomes encode two mono-functional transpeptidases (PBP2 and PBP3 (FtsI)) [25], which cross link the glycan chains via peptide bridges in free-living bacteria. They encode a DD-carboxypeptidase (PBP6a) [25] that catalyzes the removal of the terminal D-alanine from the peptide side chain. They possess as well a gene coding an

amidase (AmiA) [25] that cleaves peptide side chains from glycan chains during cell division in extracellular bacteria (Figure 49) [182].

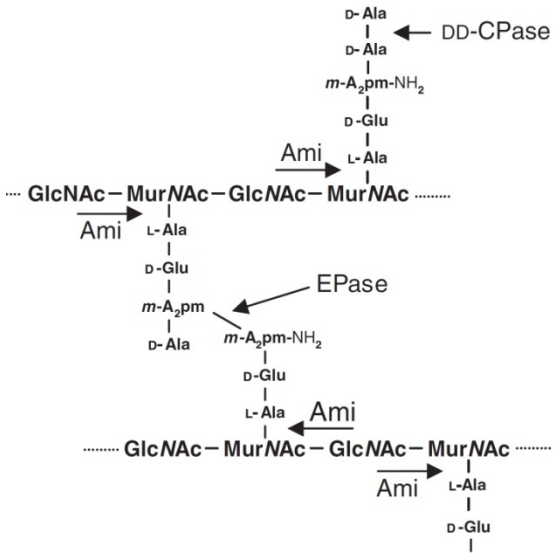

Figure 49: Schematic presentation of amidase (Ami), endopeptidase (EPase) and DD-carboxypeptidase activities on peptidoglycan (modified from [182]).

Chlamydiae are susceptible to treatment with penicillin and other β-lactam antibiotics as they harbor three PBPs homologous to PBP2, PBP3 and PBP6a from *E. coli* [27, 78, 120]. Due to expected difficulty in PBPs cloning and over-expression, the three genes were cloned in three different vectors with different promoter systems, tags and tag-positions. Cloning in pASK-IBA vectors with the tightly repressed *tetA* promoter showed higher rates of positive clones in comparison to pET-21b vector with constitutive T7 promoter. The predicted secondary structures in the PBPs genes may hinder the attachment to the restricted pET-21b vectors during ligation step. The weak expression of PBPs, with their expected cytotoxicity, induced by the constitutive T7 promoter may result in dramatic selection against the *E. coli* that harbor the recombinant vector. Additionally, good levels of protein over-production that allow successful protein purification were not achieved using the three recombinant vectors under different conditions for induction and purification. Improper folding of foreign or cytotoxic proteins is a common problem of over-expression in *E. coli* that leads to protein degradation by proteases and/or inclusion body formation. With the help of co-expression of a chaperone plasmid pKJE7 that encodes three chaperone protein (DnaK, DnaJ and GrpE), the three chlamydial PBPs were

successfully over-produced and purified. Because of the predicted transmembrane domains, the expressed proteins were solubilized with help of sodium lauroylsarcosine (0.5%). An alternative technique was to clone truncated proteins that lack the predicted transmembrane domain, which may impair the protein folding and activity. Therefore, the cloning of full-versions of the three PBPs was preferred.

The genome of *E. coli* encodes 12 PBPs, of which only PBP2, PBP3 with one of the bi-functional proteins PBP1a or PBP1b are enough to maintain the wild-type phenotype of the bacteria [113]. In the genome of *C. trachomatis*, the gene encoding PBP3 is located in an operon upstream of *murE* and *amiA* and downstream of *mraW* and the gene for the hypothetical protein CT272. In all Gram-negative bacterial *murE* and *mraW* are part of the division cell wall (dcw) cluster [183, 184]. *mraW* is an essential gene for cell division that is universally distributed in all bacteria and encodes a cytoplasmic protein with methyltransferase activity [185]. The genomic environment of the PBP3 encoding gene strongly supports its role in cell wall division in chlamydiae.

In the diploma thesis by Anna Klöckner, the *in vitro* activity of AmiA from *C. pneumoniae* to hydrolyze lipid II was detected using a dye release assay. The chlamydial protein recognized both lipid I and lipid II as a substrate. An *in vivo* interaction between chlamydial AmiA and PBP3 was detected using BACTH system analyses.

In this project, *in vitro* assays were established to test the transpeptidase activity of chlamydial PBP2 and PBP3 using natural and artificial substrates. It was not possible to demonstrate transpeptidase activity by PBP2 and PBP3 from *C. pneumoniae* under tested conditions. Optimizations for both assays are still required to test the functionality of both proteins. Utilization of a bi-functional PBP from Gram-negative origin like *E. coli,* instead of the staphylococcal protein, may allow the transglycosylation reaction to be performed at the same reaction conditions. The PG sacculi involved in the assays were from *E. coli* and *B. subtilis* origins, which offer a substrate with length and cross-linking degree that differs from the natural substrate expected for cell wall-less bacteria. PG sacculi from other bacterial origins may present a better substrate for the chlamydial proteins. Detection of lipid II attached to PG sacculi using a radioactive labeled lipid II variant [106, 149] may increase the sensitivity of the assay to detect the activity of chlamydial PBP2 and PBP3.

In a very recent study on PBP2 and PBP3 from *C. trachomatis* [170], immunofluorescent microscpic analyses of chlamydiae treated with the PBP-specific antibiotics, mecillinam or piperacillin, showed clear morphological changes indicating sensitivity of chlamydiae to both compounds [170]. While mecillinam is a selective inhibitor of the transpeptidase activity of PBP2 from *E. coli* [186-189] inducing loss of its rod shape [190], piperacillin blocks the activity of PBP3 from *E. coli* selectively [118, 188, 191, 192] resulting in filamentous *E. coli* [191]. Additionally, BACTH system analyses revealed interactions between chlamydial PBP2, PBP3, self-interaction for dimerization of PBP3 and association of PBP2 and PBP3 with the multifunctional cell division protein FtsK [170]. These findings confirm the functionality of the chlamydial HMM-PBPs and their role in coordinating cell division and recycling of lipid II.

The established assays to test the carboxypeptidase activity of the chlamydial PBP6a showed no activity under tested conditions using both natural and artificial substrates. The genome of *E. coli* encodes several DD-carboxypeptidases, PBP4, PBP5, PBP6a and PBP6b of which PBP5 is the most abundant and most powerful carboxypeptidase [33, 193]. Previous studies revealed a very weak activity for PBP4, PBP6a and PBP6b [150, 151, 194]. Despite the close sequence similarity between PBP5 and PBP6a (65% identical), the latter was unable to substitute for PBP5 function *in vivo* [195]. With the exception of PBP5, which has a clear role in maintaining cell diameter and typical morphology in *E. coli*, other carboxypeptidases seems to be dispensable and the conservation of these non-essential enzymes in *E. coli* is still questionable [193]. Two hypotheses were postulated for the function of PBP6a in *E. coli*. First: PBP6a may have a carboxypeptidase activity similar to PBP5 but with much lower efficiency, however, many observations are not consistent with this hypothesis [193]. Second: PBP6a of *E. coli* may be involved in the production of lipid II variants with tripeptide side chain [196], which is thought to be the substrate for PBP3 during septation [118, 197]. Over-expression of PBP6a can restore cell division in filamentous *E. coli* mutants due to extremely reduced levels of PBP3 expression [196]. The exact mechanism for tripeptide generation by PBP6a is poorly understood [193]. Additional studies are still required to test whether chlamydial PBP6a follows the first or second hypothesis.

Based on the previously discussed results on chlamydial proteins, the following model for the linkage between lipid II biosynthesis and cell division in chlamydiae could be proposed (Figure 50). MreB interacts with MurF, MraY and MurG and serves as a scaffold directing lipid II biosynthesis to the septum. The synthesized precursor is processed by a rudimentary cell wall biosynthesis-cell division machinery. Lipid II is translocated across the membrane by the activity of the flippase FtsW. Subsequently, PBP2, PBP3 and AmiA are involved in catalyzing reactions that result in unknown products and the release of the bactoprenol carrier. During such a process, a rudimentary (glycan-less [78]) by product, in which the peptide side chains are cross-linked by peptide bonds, might result. Recycling of C_{55}-P lipid carrier is unclear in the absence of pyrophosphorylases (YeiU, YbjG, PgpB and UppP) that have been described so far to dephosphrylate C_{55}-PP in *E. coli*.[54]

It should be noted that 28% of the predicted proteins from the chlamydial genome sequencing showed no sequence similarity to any of known proteins [25], therefore, no clear function could be assigned for these proteins. Analysis of the functionality of these unknown proteins may offer better elucidation of the chlamydial anomaly.

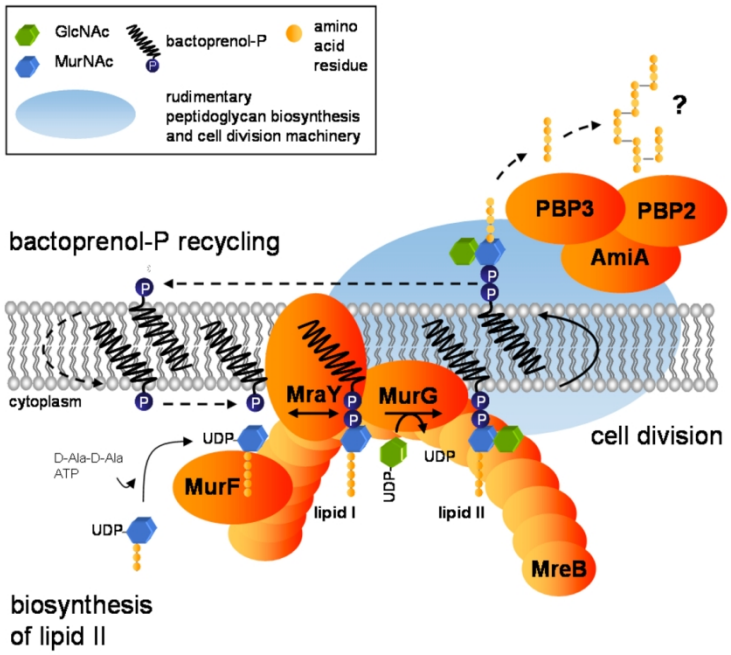

Figure 50: Proposed model for co-ordination of lipid II biosynthesis and cell division in chlamydiae

A complete cycle of lipid II biosynthesis and processing, including translocation to the outside and recycling of the bactoprenol carrier needs to be maintained for coordinated function of the cell division machinery (which may consist of FtsW, FtsI, FtsH, FtsK and FtsY). The actin homolog MreB functionally organizes MurF, MraY and MurG and directs lipid II biosynthesis to the septum. The synthesized precursor is translocated to the outside where the release of the bactoprenol carrier is catalyzed through the activity of FtsI, PBP2 and AmiA.

Question marks and dashed arrows indicate steps of the proposed pathway that remain to be elucidated (GlcNAc: N-acetylglucosamine; MurNAc: N-acetylmuramic acid)

Orchestration of lipid II biosynthesis and processing as well as cell division may be achieved by formation of multi-enzyme complexes. Additional studies on the interaction of MreB with proteins involved in these fundamental processes will be helpful for a better understanding into the role of MreB in functionally organizing lipid II biosynthesis at the septum and as such in maintaining functional division machinery. Deeper insight in co-ordination of lipid II biosynthesis and cell division will contribute to elucidate the inscrutable chlamydial anomaly at a molecular level. In addition, it will provide a basis for the design of new antibacterial drugs to combat diseases caused by these major intracellular pathogens.

6 SUMMARY AND CONCLUDING REMARKS

Chlamydiae are Gram-negative, obligate intracellular pathogens. Because of the isotonic intracellular niche of the host, a functional cell wall is dispensable in these endobacteria. Surprisingly, a nearly complete pathway for the biosynthesis of cell wall precursor lipid II is conserved in chlamydiae. Moreover, the organism is susceptible to penicillin and other antibiotics that inhibit cell wall biosynthesis. This paradox, which is known as chlamydial anomaly, raises the question about the essential role of cell wall building block lipid II in maintaining bacterial growth and replication.

Cell wall biosynthesis and cell division in prokaryotes are driven by tightly coordinated, partially overlapped machineries. Therefore, it was hypothesized that lipid II, which is an essential part of both processes, is required to maintain a functional divisome in chlamydiae.

The bacterial actin homolog MreB, which is found exclusively in non-spherically shaped bacteria, is considered to organize the incorporation of cell wall precursors into the side-wall, whereas the tubulin homolog FtsZ, which is ubiquitously distributed in almost all bacteria, is known to tether incorporation of cell wall building blocks at the developing septum. Unexpectedly, chlamydial genomes lack the essential central organizer of cell division *ftsZ*, but harbor despite of its spherical shape the rod-shape determining *mreB*.

In this project, it was investigated how chlamydiae can divide in absence of FtsZ. It was demonstrated that cytoskeletal protein MreB might compensate for FtsZ for maintaining a functional divisome machinery in chlamydiae. It was shown that chlamydial MreB polymerizes *in vitro* and that polymerization is not inhibited by the blocking agent A22. As observed for MreB from *B. subtilis*, chlamydial MreB does not require ATP for polymerization but is capable of ATP hydrolysis in phosphate release assays. Co-pelleting and bacterial two-hybrid experiments indicate that MreB from *C. pneumoniae* interacts with MurF, MraY and MurG, three key components in lipid II biosynthesis. In addition, MreB polymerization is improved in the presence of MurF. These findings suggest that MreB is involved in tethering biosynthesis of lipid II and as such may be necessary for maintaining functional divisome machinery in *Chlamydiaceae*.

While chlamydial genomes lack the *alr* gene encoding for alanine racemase that is responsible for D-alanine synthesis in other bacteria, several findings suggest that D-alanine should be part of chlamydial lipid II. Preliminary results on the racemization activity of the chlamydial GlyA show that the protein may provide D-alanine in chlamydiae.

The sensitivity of chlamydiae to penicillin and other β-lactam antibiotics is attributed to the presence of three PBPs encoded in the chlamydial genome. Chlamydiae have three PBPs homologous to PBP2, 3 and 6a from *E. coli.* PBP2 and PBP3 are essential mono-functional transpeptidases in *E. coli*. On the other hand, the *E. coli* PBP6a shows a weak *in vitro* carboxypeptidase activity. To get insights into the functionality of the chlamydial PBPs, the three proteins were cloned, over-produced and purified successfully. Further optimizations for the established assays are still required to get clear insights into the functionality of these proteins in *Chlamydiaceae*.

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LIST OF PUBLICATIONS

Parts of this work were shortly published or presented in the following

Original articles:

- **Gaballah A,** Kloeckner A, Otten C, Sahl HG, Henrichfreise B. Functional analysis of the cytoskeleton protein MreB from Chlamydophila pneumoniae. PLoS One 2011, 6:e25129.

Congress presentations

- **Gaballah A**, Klöckner A, Otten C, Bühl H, De Benedetti S, Schneider T, Sahl HG, Henrichfreise B. Peptidoglycan biosynthesis in cell wall-lacking bacteria: news on the chlamydial anomaly. 10th German Chlamydiae workshop. Erfurt 4th-6th April 2012. (talk)
- **Gaballah A**, Klöckner A, Otten C, Bühl H, De Benedetti S, Schneider T, Sahl HG, Henrichfreise B. Cell wall biosynthesis in cell wall-less endobacteria - new options for antibiotic treatment. 10th German Chlamydiae Workshop. Erfurt 4th-6th April 2012.
- De Benedetti S, **Gaballah A**, Sahl HG, Henrichfreise B. Functional analysis of the translation elongation factor EF-Tu from *Chlamydophila pneumoniae*. Annual conference of the German Society for Hygiene and Microbiology (DGHM), Essen, 25th – 28th September 2011.
- **Gaballah A**, Klöckner A, Otten C, Sahl HG, Henrichfreise B. Functional analysis of the cytoskeleton protein MreB from *Chlamydophila pneumoniae.* International Symposium of the International Graduate Research School (Biotech Pharma), Bonn. 16th-17th September 2011.
- Klöckner A, **Gaballah A**, Sahl HG, Henrichfreise B. Analysis of the chlamydial amidase AmiA. Annual conference of the Association of General and Applied Microbiology (VAAM), Karlsruhe. 3rd-6th April 2011.

- Henrichfreise B, **Gaballah A**, Pöllinger C, Klöckner A, Otten C, Sahl HG. Peptidoglycan biosynthesis and cell division in cell wall-lacking bacteria. 5th Biennial Meeting of the Chlamydia Basic Research Society. Redondo Beach, CA, 18th -21st March 2011. (Talk)
- **Gaballah A,** Klöckner A, Schneider T, Müller A, Sahl HG, Henrichfreise B. Peptidoglycan biosynthesis and cell division in cell wall-lacking bacteria. German Chlamydiae Workshop. Ascona, Switzerland. 22nd -25th February 2011. (Talk)
- Henrichfreise B, **Gaballah A**, Poellinger C, Kloeckner A, Otten C, Sahl HG. Peptidoglycan biosynthesis and cell division in cell wall-lacking bacteria. Gordon Research Conference: Bacterial Cell Surfaces, New London, NH, 27th June- 2nd July 2010.
- Henrichfreise B, **Gaballah A**, Pöllinger C, Klöckner A, Otten C, Sahl HG. Analysis of the cytoskeleton protein MreB from *Chlamydia pneumonia*. Annual Joint Conference of the Association for General and Applied Microbiology (VAAM) and the German Society for Hygiene and Microbiology (DGHM), Hannover. 28th - 31st March 2010.
- Henrichfreise B, **Gaballah A**, Pöllinger C, Klöckner A, Otten C, Sahl HG. Analysis of the chlamydial cytoskeleton protein MreB. 8th German Chlamydiae Workshop München, 24th -26th February 2010.

ERKLÄRUNG

An Eides Statt versichere ich, dass ich die vorliegende Arbeit einschließlich beigefügter Zeichnungen und Darstellungen selbstständig und nur mit den angegebenen Hilfsmitteln angefertigt habe. Die Stellen der Arbeit, die anderen Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind unter Angabe der Quellen als Entlehnung kenntlich gemacht.

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Bonn, im Juli 2012

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Ahmed Hassan Ahmed Gaballah