Wolbachia, endosymbionts of arthropods and filarial nematodes: Requirement of cell wall biosynthesis for cell division And Replication in a host cell-free culture

Dissertation

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I. Abstract

Obligate intracellular endobacteria of the genus *Wolbachia* are widespread in arthropods and filarial nematodes. In disease-causing filarial nematode species, responsible for over 150 million cases of human filariasis in tropical regions, these endobacteria are essential for the survival of the worm. *Wolbachia* depletion by antibiotics is an effective approach for anti-filarial therapy. But the development of drugs suitable for mass drug administration is of urgent need.

A specific target for novel antibiotic discovery is the bacterial cell wall since eukaryotes lack an orthologous structure. Thus, the discovery of genes involved in cell wall metabolism in the cell wall-less *Wolbachia* opened promising possibilities. In this PhD project, insights into the structure of the cell wall precursor lipid II are provided in *Wolbachia* and a lipoprotein was identified as a potential interaction partner of the molecule. Strikingly, it was found that inhibition of lipid II biosynthesis resulted in impaired cell division in *Wolbachia*. These results confirmed the biological necessity of lipid II in *Wolbachia* and indicate a possible secondary function of this molecule in cell wall containing bacteria. In contrast, division defects were not observed subsequent to the depletion of the major cell division protein FtsZ, yielding an uncertain role of FtsZ in cell division of *Wolbachia*.

The investigation of obligate intracellular *Wolbachia* endobacteria is extremely challenging. In a second part of this PhD thesis the requirements for *Wolbachia* growth in a host cell-free *in vitro* culture system were characterized. The supplementation of a cell lysate derived from an insect cell culture facilitated extracellular *Wolbachia* replication and the bacteria remained viable and infective in this culture for at least 12 days. Furthermore, evidence was given for the growth promoting factors necessary for host cell-free cultivation. In conclusion, intracellular *Wolbachia* are able to replicate outside their host cell and further steps for the establishment of an extracellular culture system as a powerful tool for molecular biological approaches are discussed.

II. Abbreviations

- *A. albopictus*: *Aedes albopictus*
- ADEP: Acyldepsipeptide
- Alr: Alanine racemase
- AmiD: N-acetylmuramoyl-L-alanine amidase, family 2
- AVG: L-aminoethoxyvinylglycine
- *B. malayi*: *Brugia malayi*
- *B. stearothermophilus*: *Bacillus stearothermophilus*
- bp: base pair(s)
- BrdU: Bromodeoxyuridine
- BSA: Bovine serum albumin
- *C. crescentus*: *Caulobacter crescentus*
- DAAO: D-amino acid oxidase from porcine kidney
- D-ala: D-alanine
- DapE: Succinyl-diaminopimelate desuccinylase
- DapF: Diaminopimelate epimerase
- Ddl: D-alanine–D-alanine ligase
- DEC: Diethylcarbamazine
- D-glu: D-glutamate
- *E. coli*: *Escherichia coli*
- FCS: Fetal calf serum
- Fts: Filamentous temperature-sensitive
- FtsA: Cell division protein FtsA
- FtsE: Cell division ATP-binding protein FtsE
- FtsK: DNA translocase FtsK
- FtsW: : Lipid II flippase FtsW (Cell division protein FtsW)
- FtsX: Cell division protein FtsX
- FtsZ: Cell division protein FtsZ
- GlcNAc: *N*-acetylglucosamine
- GlyA: Serine hydroxymethyltransferase
- L-ala: L-alanine

- LpoA: Penicillin-binding protein activator LpoA
- LpoB: Penicillin-binding protein activator LpoB
- *meso*-Dap: *meso*-diaminopimelic acid
- MetC: Cystathionine beta-lyase
- MraY: UDP-MurNAc-pentapeptide phosphotransferase
- MreB: Rod shape-determining protein MreB
- MreC: Rod shape-determining protein MreC
- MreD: Rod shape-determining protein MreD
- MurA: UDP-N-acetylglucosamine 1-carboxyvinyltransferase
- MurB: UDP-N-acetylenolpyruvoylglucosamine reductase
- MurC: UDP-N-acetylmuramate--L-alanine ligase
- MurD: UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase
- MurE: UDP-N-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase
- MurF: UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase
- MurG: UDP-GlcNAc-undecaprenoyl-pyrophosphoryl-MurNAc-pentapeptide transferase
- MurI: Glutamate racemase
- MurNAc: *N*-acetylmuramic acid
- Pal: Peptidoglycan-associated outer membrane lipoprotein Pal
- PBP2: Penicillin-binding protein 2
- PBP3: Penicillin-binding protein 3
- PBP6a: D-alanyl-D-alanine carboxypeptidase
- PCR: Polymerase chain reaction
- PEP: Phosphoenolpyruvate
- PLP: Pyridoxal phosphate
- qPCR: Quantitative real-time PCR
- RodA: Rod shape-determining protein RodA
- RodZ: Cytoskeleton protein rodZ
- RT-PCR: Reverse transcriptase PCR
- SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- SEDS protein family: shape, elongation, division and sporulation protein family
- SEM: Standard error of the mean

- TEMED: Tetramethylethylenediamine
- TolA: Membrane anchored protein TolA
- TolB: Translocation protein TolB
- TolQ: Membrane anchored protein TolQ
- TolR: Membrane anchored protein TolR
- UDP-MurNAc-pentapeptide:Uridine diphosphate-*N*-acetylmuramic acid-pentapeptide
- *w*Alb B: *Wolbachia pipientis* strain of *Aedes albopictus* B
- *w*Bm*: Wolbachia* endosymbiont of *Brugia malayi* (NCBI RefSeq NC_006833.1)
- WHO: World Health Organization
- *w*Mel: *Wolbachia pipientis* endosymbiont of *Drosophila melanogaster* (NCBI RefSeq NC_002978)
- *w*Pa: *Wolbachia pipientis* endosymbiont of *Culex quinquefasciatus pel* (NCBI RefSeq NC_010981.1)
- *w*PAL: *Wolbachia* peptidoglycan associated lipoprotein (PAL)
- *w*Ri: *Wolbachia pipientis* endosymbiont of *Drosophila simulans* strain Riverside (NCBI RefSeq NC_012416)
- ZapA: Cell division protein ZapA
- ZipA: Cell division protein ZipA

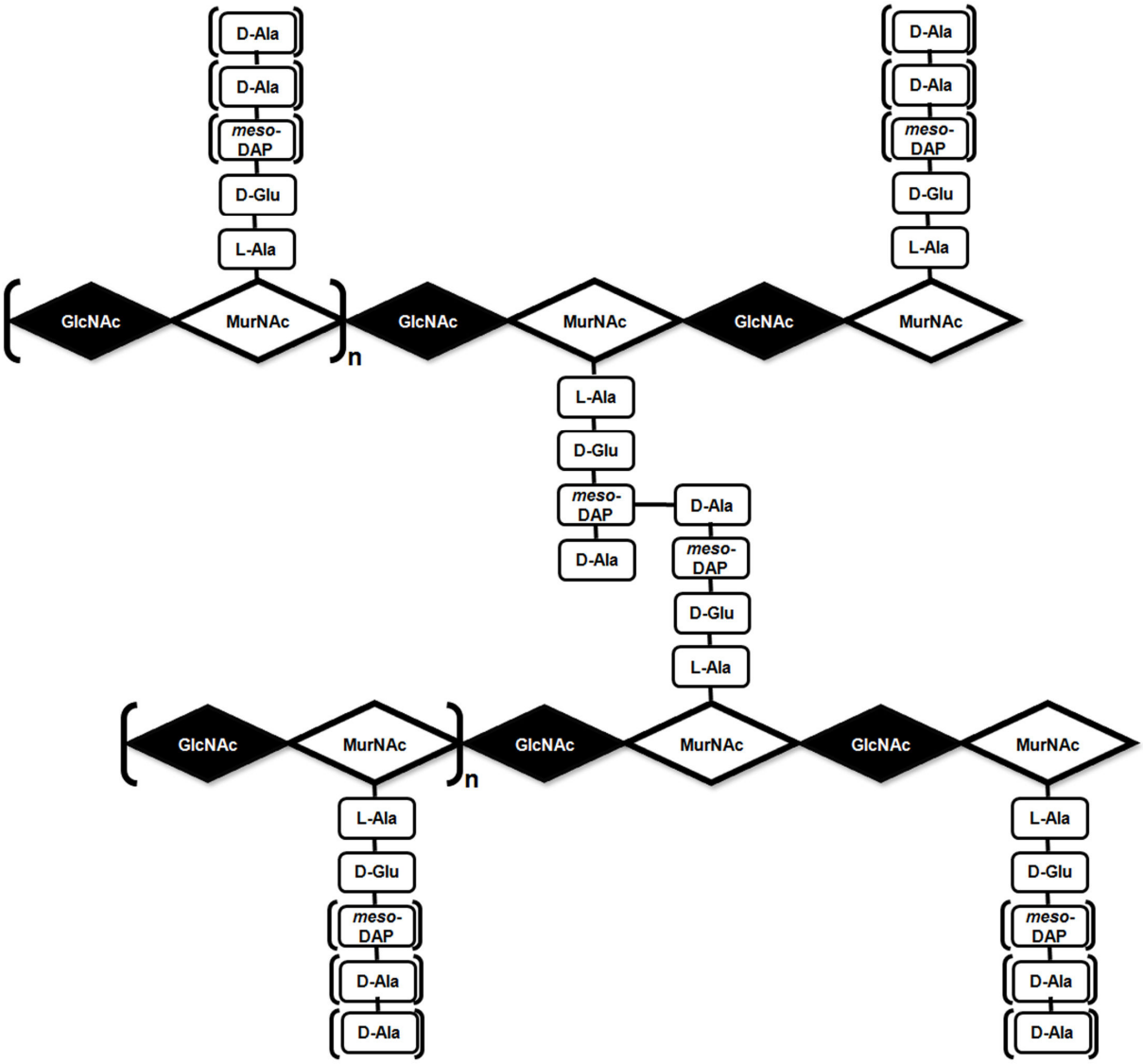
1 Introduction

1.1 Peptidoglycan metabolism in Gram-negative bacteria

1.1.1 Synthesis of a stress-bearing peptidoglycan sacculus

Free-living bacteria are dependent on a cell surrounding peptidoglycan sacculus to withstand internal osmotic pressure. In Gram-negative bacteria this stabilizing peptidoglycan is located in the periplasm between the inner and the outer lipid membranes. The sacculus is built of long, linear glycan chains that are cross-linked by short peptides (Figure 1.1). The glycan chains are composed of alternating β 1,4 linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) molecules. Short peptides of five amino acids are attached to the carboxy group of the MurNAc molecule generally composed of D-amino acids. In the mature peptidoglycan, amino acids are cleaved off the pentapeptide side chain and most side chains appear as di-, tri-, or terapeptides (Vollmer, W. *et al.* 2008a). Cross-linking of the peptides occurs commonly between the amino acid at position 4 and position 3 of two peptides. While the structure of the glycan chains is mostly conserved between different bacterial species, the composition and cross-linkage of the peptides is variable (Vollmer, W. *et al.* 2008b). L-alanine at position 1 of the peptide side chain is the only amino acid conserved in all bacterial species. In most Gram-negative bacteria the pentapeptide is composed of L-alanine – D-glutamate – *meso*-diaminopimelic acid (*meso*-DAP) – D-alanine – D-alanine. The unusual amino acid *meso*-DAP, an intermediate product of the bacterial lysine biosynthesis pathway, is characteristically found in the peptidoglycan of Gram-negative bacteria.

Figure 1.1: The basic structure of peptidoglycan in Gram-negative bacteria (adapted from Park and Uehara 2008 (Park, J. T. *et al.* 2008)). The sugar chains of peptidoglycan are built of alternating GlcNAc and MurNAc molecules and are cross-linked by short peptide bridges. Free peptides can be present as di-, tri-, tetra-, or pentapeptides as depicted by brackets.

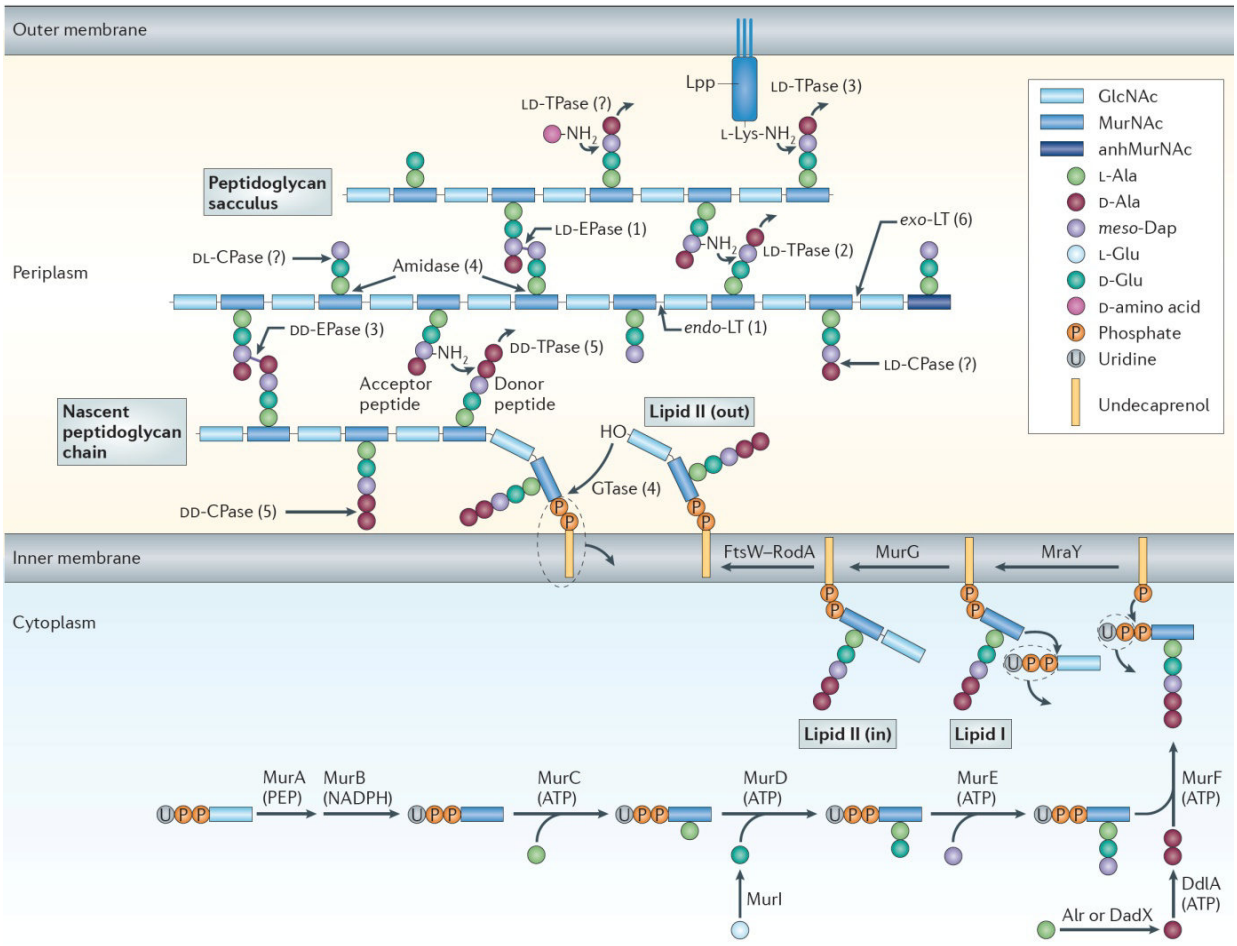


The biosynthesis of peptidoglycan starts in the cytoplasm where the six enzymes MurA to MurF catalyze the formation of uridine diphosphate - *N*-acetylmuramic acid - pentapeptide (UDP-MurNAc-pentapeptide) from UDP-GlcNAc (Figure 1.2). In the next step the phospho-MurNAc-pentapeptide moiety is attached to the membrane-bound lipid carrier undecaprenyl phosphate by the enzyme MraY, yielding lipid I. The lipid I molecule is further processed to the peptidogylycan precursor lipid II by the addition of a molecule of GlcNAc by the enzyme MurG. In the next step lipid II is flipped across the cytoplasmic membrane into the periplasm. It was recently demonstrated that the transmembrane protein FtsW is such a transporter for lipid II across the membrane. The highly related proteins RodA and SpoVE compose together with FtsW the shape, elongation, division and sporulation (SEDS) protein family and are most probably lipid II flippases, as well

(Mohammadi *et al.* 2011). In the periplasm, lipid II is linked to the nascent glycan chains by glycosyltransferases. Transpeptidases catalyze the formation of peptide cross-links while the degree of cross-linking is regulated by carboxypeptidases that cleave amino acids off the peptide side chains. The transpeptidases and carboxypeptidases belong to the family of penicillin binding proteins (PBPs) named after their capacity to covalently bind penicillin (Suginaka *et al.* 1972). Some PBPs also exhibit glycosyltransferase activity and are therefore termed bifunctional PBPs. The monofunctional glycosyltransferases do not belong to the PBP protein family (Holtje 1998).

The insertion of a new peptidoglycan strand into the existing sacculus requires the degradation of mature peptidoglycan (Vollmer, W. *et al.* 2008a). The breakdown is mediated by three classes of lytic enzymes: endopeptidases that cleave the peptide cross-links, amidases that cut the bond between the sugar and the peptide moieties and lytic transglycosylases that cleave the glycosidic bond between GlcNAc and MurNAc (Park, J. T. *et al.* 2008) (Figure 1.2). This degradation process is essential for proper cell division in which septal peptidoglycan needs to be cleaved to allow cell separation. As a consequence, as much as 40 – 50 % of the sacculus in *Escherichia coli* is removed during each generation time. The degradation products are recycled, translocated into the cytoplasm via permeases and recovered by several recycling enzymes to be available again for the synthesis of new peptidoglycan (Park, J. T. *et al.* 2008).

Figure 1.2: Peptidoglycan metabolism in Gram-negative bacteria [Adapted from (Typas *et al.* 2011)]. The peptidoglycan precursor lipid II is synthesized in the cytoplasm and flipped across the cytoplasmic membrane most likely by FtsW or RodA. The sporulation specific potential flippase SpoVE is not shown. A nascent peptidoglycan chain is formed from lipid II molecules by the action of glycosyltransferases (GTAse) and linked to the sacculus by DD-transpeptidases (DD-TPase). The peptide moieties are trimmed by DD-, LD- and DL-carboxypeptidases (CPases). Cleavage is catalyzed by three classes of lytic enzymes, DD- and LD-endopeptidases (EPases) that cleave peptide cross-links, amidases that remove the peptides from the glycan chains and lytic transglycosylases (LTs) that cleave the glycan chains. LD-TPases promote the attachment to the major outer membrane lipoprotein Lpp by LD-cross links and the binding of unusual D-amino acids. The numbers of enzymes in *E. coli* that are known until today are shown in brackets.



The peptidoglycan layer is associated with several proteins, which most of are peptidoglycan processing enzymes (Buist *et al.* 2008). Only one protein is known to be covalently bound to peptidoglycan, Braun´s lipoprotein (Lpp) (Vollmer, W. *et al.* 2008b). Lpp is highly abundant in the outer membrane and promotes membrane integrity by connecting the outer membrane and the peptidoglycan layer. Another lipoprotein of the outer membrane that facilitates this connection is the peptidoglycan associated lipoprotein (Pal). This protein interacts with peptidoglycan non-covalently and requires the amino acid *meso*-DAP for its binding (Parsons *et al.* 2006). It furthermore interacts with the transmembrane protein TolA that, together with the two other transmembrane proteins TolQ and TolR, forms a sub-complex in the inner membrane (Figure 1.3). The periplasmic protein TolB competes with peptidoglycan to bind Pal and therefore regulates this association. The membrane spanning Tol-Pal complex also interacts with other outer membrane proteins like Lpp via Pal and altogether results in a transenvelope connection of the outer membrane, inner membrane and peptidoglycan layer, thus

facilitating membrane integrity (Godlewska *et al.* 2009). However, the Tol-Pal complex was not only shown to play a role in cell wall stabilization but also to be involved in cell division (see section 1.1.2).

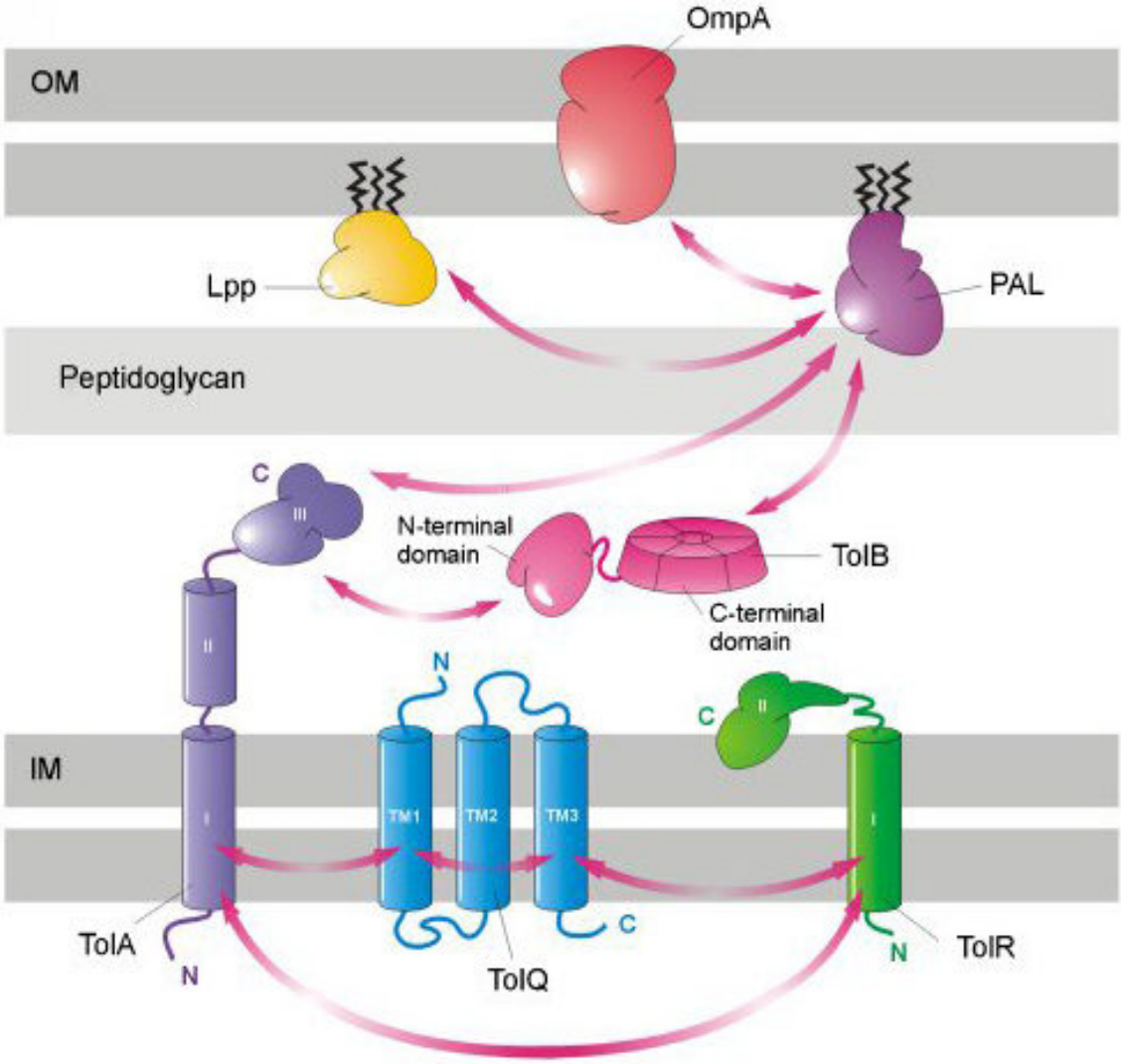


Figure 1.3: Localization and interactions of the Tol-Pal complex in Gram-negative bacteria (Godlewska *et al.* 2009). Interactions are depicted by arrows. The Pal protein, located in the outer membrane, binds to peptidoglycan and interacts with TolA in the inner membrane that is associated with the transmembrane proteins TolQ and TolR. TolB is located in the periplasm and acts as a regulator for the binding of Pal to peptidoglycan. Pal furthermore interacts with other outer membrane proteins like Lpp and OmpA.

1.1.2 Functional distinction: The elongasome and the divisome

The enzymes of the lipid II biosynthesis pathway are conserved among bacterial species and exhibit a unique function. However, those enzymes catalyzing the late, periplasmic steps of peptidoglycan synthesis are in most cases redundantly present, e.g. in *E. coli* five transpeptidases and four glycosyltransferases are found (Figure 1.2). Many of those late peptidoglycan synthesizing enzymes were shown to exhibit cell cycle specific

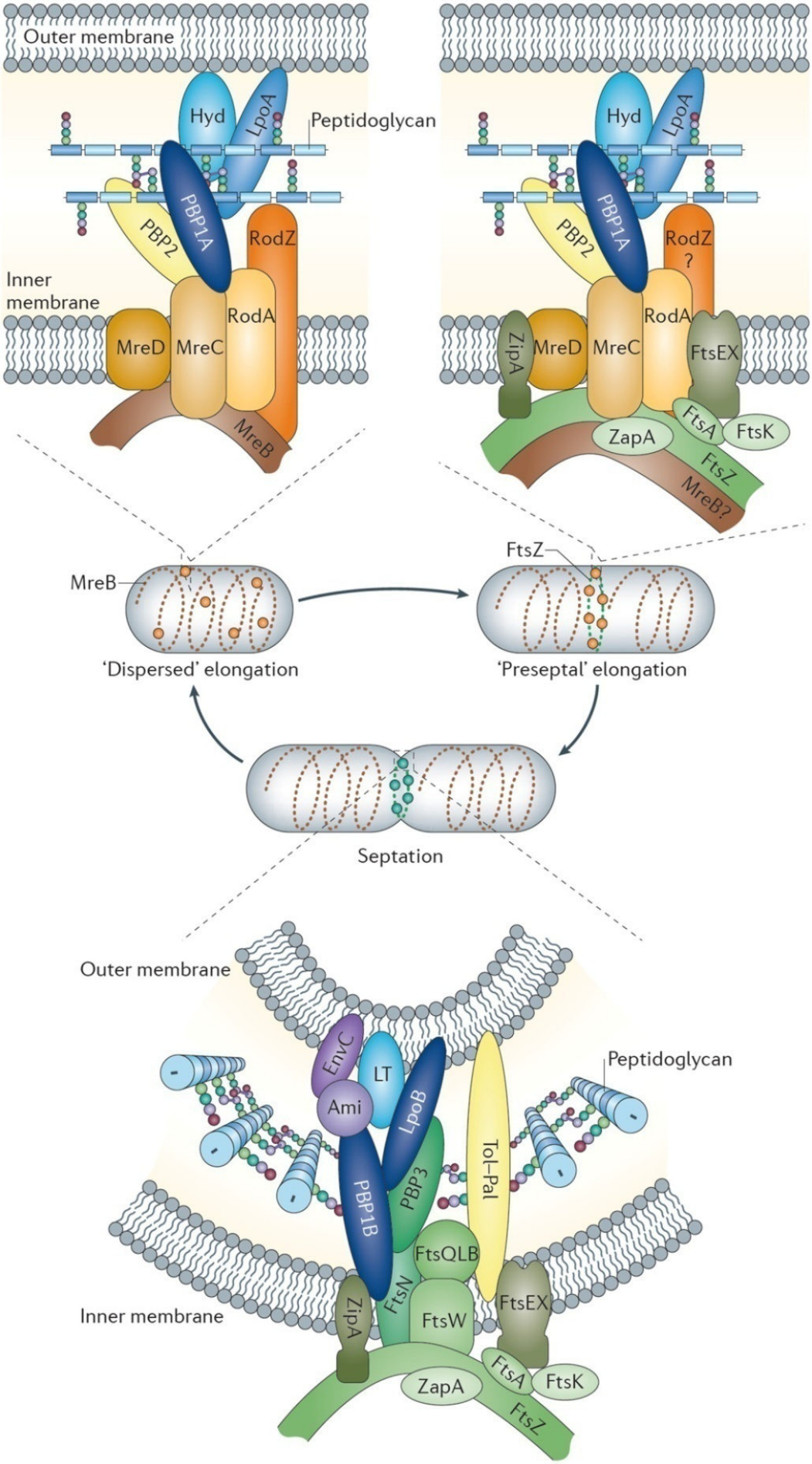
functions, i.e. they act either during cell elongation or during cell division (Scheffers *et al.* 2005). Especially in rod-shaped bacteria like *E. coli* the functional differentiation is reflected by the localization of the enzyme since nascent peptidoglycan is inserted into the mature sacculus in different modes (Typas *et al.* 2011). During growth the sites of elongation specific peptidoglycan synthesis are dispersed over the whole cell surface whereas in preseptal and septal cell stages the elongation specific mode of synthesis switches to a division specific mode that takes place at midcell until cell division is completed (Figure 1.4).

Previous examinations indicated a direct interaction of elongation specific enzymes and division specific enzymes, respectively. It could be shown for instance that the transpeptidase PBP2 in association with the flippase RodA is involved in cell elongation while the subcomplex of the transpeptidase PBP3 and the flippase FtsW is located at the division site (de Pedro *et al.* 2001; Datta *et al.* 2006; den Blaauwen *et al.* 2008; Fraipont *et al.* 2010; White *et al.* 2010). The organization and localization of such subcomplexes is controlled by cytoskeletal proteins. The elongation specific complex is coordinated by MreB which forms cytoplasmic, membrane-attached helices that surround the whole cell while the division specific proteins are recruited by the cell division protein FtsZ (den Blaauwen *et al.* 2008; White *et al.*; Typas *et al.* 2011). FtsZ polymerizes at midcell to form the Z-ring thus initiating the cell division process. The polymerization is tightly regulated by several factors that are synthesized dependent on the stage of the cell cycle, DNA damage, metabolic or oxidative stress, and the phase of division (Kirkpatrick *et al.* 2011). In the early stage of Z-ring formation many proteins that promote stabilization and membrane attachment of the ring structure are recruited midcell by FtsZ. After this step the peptidoglycan synthesizing enzymes are assembled to facilitate septum formation (Margolin 2005). This division mediating protein complex is furthermore associated with the membrane spanning Tol-Pal complex (see section 1.1.1). This complex was shown to be required for proper outer membrane invagination during cell division in *E. coli* and *Caulobacter crescentus* and it was proposed that the interactions of Pal and the Tol proteins actively facilitate the invagination process (Gerding *et al.* 2007; Yeh *et al.* 2010). In this model the Pal protein that is located in the outer membrane alternately interacts with peptidoglycan and the TolA protein of the Tol-complex which is

located in the inner membrane. Due to the binding of TolA, Pal is pulled inward thus leading to the invagination of the outer membrane.

Based on these collective discoveries of the interactions and associations of elongation or division mediating proteins it is currently assumed that two distinct huge and tightly coordinated multi-protein complexes exist, the divisome and the elongasome. Cell elongation is facilitated by the elongasome which consists of peptidoglycan synthesizing and hydrolyzing enzymes as well as cytoskeletal and regulating proteins, while cell division is mediated by homologous as well as division specific proteins forming the divisome (Figure 1.4).

Figure 1.4: Different multi-protein complexes facilitate the synthesis of peptidoglycan during the cell cycle of *E. coli* (Typas *et al.* 2011). During cell growth lipid II is flipped into the periplasm by RodA. Peptidoglycan is synthesized by the elongation specific enzymes PBP1A and PBP2 that are regulated by LpoA. These enzymes are organized by the cytoskeletal protein MreB and its associated proteins MreC, MreD and RodZ. To insert newly formed peptidoglycan, the sacculus is cleaved by yet unknown hydrolases (Hyd). In the preseptal stage FtsZ polymerizes to form the Z-ring mid cell and is associated with ZapA, ZipA, FtsA, FtsEX and FtsK. Due to the action of these proteins, the elongation specific complex is re-located to midcell. It is not known if MreB participates at this time point. During septation lipid II is translocated via FtsW and peptidoglycan is synthesized by PBP1B and PBP3, which are regulated by LpoB. Division is mediated by the Fts-proteins and the Tol-Pal complex and septum cleavage is promoted by lytic transglycosylases (LT) and amidases (Ami).


Outer membrane
Hyd
LpoA
Peptidoglycan
PBP1A
PBP2
RodZ
Inner membrane
RodA
MreD
MreC
MreB
Hyd
LpoA
PBP1A
PBP2
RodZ
?
RodA
ZipA
MreD
MreC
FtsEX
FtsA
FtsK
ZapA
FtsZ
MreB?
MreB
'Dispersed' elongation
FtsZ
'Preseptal' elongation
Septation
Outer membrane
EnvC
LT
Ami
LpoB
Tol–Pal
Peptidoglycan
PBP1B
PBP3
FtsQLB
FtsN
FtsW
FtsEX
ZipA
Inner membrane
ZapA
FtsA
FtsK
FtsZ

1.2 *Wolbachia*, endobacteria of arthropods and filarial nematodes

Intracellular α-proteobacteria of the genus *Wolbachia* are widespread in many arthropods as well as in filarial nematodes (Taylor *et al.* 1999; Fenn *et al.* 2006b; Zug *et al.* 2012). Inside the host they are found in some somatic tissues as well as in cells of the host germline and they are vertically transmitted from the mother to the offspring (Casiraghi *et al.* 2007; Serbus *et al.* 2007). Intracellularly the bacteria reside in Golgi-related vacuoles in the cytoplasm mainly associated with microtubules or the cell cortex (Cho *et al.* 2011). A common feature of endosymbiotic bacteria is the reduction of genome size due to the evolutionary adaption to their host. This is also the case in *Wolbachia* which possess only limited metabolic capacity. They lack almost all biosynthetic pathways to produce amino acids and retained only incomplete pathways for the synthesis of certain vitamins and cofactors. Those amino acids, vitamins and cofactors, which cannot be synthesized by *Wolbachia*, are most probably provided by their host (Wu, M. *et al.* 2004; Foster *et al.* 2005; Slatko *et al.* 2010).

Wolbachia of arthropods are facultative endosymbionts that exhibit a more parasitic association to their host. Although they are obligate intracellular bacteria, survival of the host cell is not necessarily dependent on the endobacteria. Therefore stability of *Wolbachia* transmission is ensured by reproductive manipulations like male-killing, induction of parthenogenesis and reproductive incompatibility between infected and uninfected organisms (Fenn *et al.* 2006b). In contrast, *Wolbachia* of filarial nematodes are obligate, mutualistic endosymbionts that are also dependent on metabolic compounds produced by the host but they in turn provide other metabolic products that are essential for worm survival, e.g. the nematode host is not able to generate purine, pyrimidine, FAD and riboflavin and thus needs to obtain these compounds by external sources or from their endosymbiont that is still able to synthesize them (Foster *et al.* 2005; Slatko *et al.* 2010). Furthermore, it has also been shown that the worm is most probably dependent on heme from *Wolbachia* (Wu, B. *et al.* 2009; Strübing *et al.* 2010). Thus, *Wolbachia* of nematodes are, in contrast to *Wolbachia* of arthropods, intrinsically tied to their host.

1.2.1 *Wolbachia* as target for anti-filarial therapy

The human filarial nematode infections onchocerciasis (river blindness) and lymphatic filariasis are endemic in tropical regions of the world and are a major disease burden on the African continent. The parasites are transmitted by an arthropod vector which releases filarial L3 larvae during a blood meal. In the human host the larvae develop to adult worms which produce and release microfilariae that are taken up again by the arthropod vector. Inside the vector the microfilariae pass through L1 and L2 larval stages to develop to L3 larvae (Wenk *et al.* 2003).

Onchocerciasis is caused by filaria of the species *Onchocerca volvulus* which is transmitted by blackflies of the genus *Simulium*. About 37 million people in Africa as well as in some spots of South and Central America are affected by this disease (Taylor *et al.* 2010). The adult worms reside in subcutaneous nodules and released microfilariae migrate through the skin and the eyes (Figure 1.5 A). The death of microfilariae induces strong immune responses that can result in severe pathologies like skin disease and blindness. About 120 million people are infected with lymphatic filariasis that is mainly caused by *Wuchereria brancrofti* and in restricted areas of Southeast Asia by *Brugia malayi* and *Brugia timori*. The L3 larvae of these species are transmitted by mosquitoes. The adult worms reside in nests within dilated lymphatic vessels mostly of the extremities and male genitalia and released microfilariae migrate into the blood stream. Due to the death of adult worms in the lymphatic vessels severe pathology occur like hydrocele in about 21 % and lyphedema can occur in about 12 % in infected individuals (WHO 2010) (Figure 1.5 B).

Several mass drug administration programs coordinated by the WHO act to stop transmission and to eliminate filariasis (Hoerauf *et al.* 2011). In this chemotherapeutic approach, three anti-filarial drugs are currently used that are administered once a year. Against onchocerciasis the drug ivermectin is used while lymphatic filariasis is treated with diethylcarbamazine (DEC) in combination with albendazole. However, in patients suffering from onchocerciasis DEC treatment can result in severe adverse reactions due to a rapid killing of filaria that in turn induces strong immune responses. Therefore DEC cannot be administered in regions that are co-endemic for both diseases and ivermectin in combination with albendazole is administered instead (Taylor *et al.* 2010). The complete elimination of lymphatic filariasis and onchocerciasis from the African continent is

complicated by several challenges. One major problem of currently used anti-filarial drugs is that all of them mainly only have mircofilariacidal effects and do not act against the adult worms. Since adult worms can survive up to 15 years (onchocerciasis) and 6 years (lymphatic filariasis) (Hoerauf A 2007), it is necessary to assure the administration of these drugs over many years. To control disease transmission in endemic countries a treatment coverage of at least 65 % is necessary and for elimination a coverage of 80 % needs to be achieved (WHO 2011). But in several areas continuous drug administration is not possible due to an unstable security situation or the lack of strong national programs (Bockarie *et al.* 2010; Mackenzie *et al.* 2011). Moreover, in regions endemic for onchocerciasis and with high initial force of transmission it has been discovered recently that even after 17 years of annual treatment transmission is likely to reappear if administration is stopped (Katabarwa *et al.* 2011). Additionally, ivermectin or DEC treatment in regions that are co-endemic for the filarial nematode species *Loa loa* can result in progressive neurologic decline and encephalopathy, thus restricting mass drug administrations in these areas (Kamgno *et al.* 2009; Bockarie *et al.* 2010; Mengome *et al.* 2010). For the successful elimination of filarial infections novel drugs are needed that act against the adult worms, which is necessary to shorten the treatment periods and to reliably stop transmission, and that are applicable in regions co-endemic for *Loa Loa*.

Filarial *Wolbachia* endosymbionts are, in contrast to arthropod *Wolbachia,* species specific. It could be shown that the depletion of nematode endosymbiont *Wolbachia* by the antibiotic doxycycline led to the sterility and degeneration of adult worms (Bockarie *et al.* 2010; Hoerauf *et al.* 2011) (Figure 1.5 C), thus demonstrating that *Wolbachia* are an effective target for anti-filarial therapy. Doxycycline does not affect *Loa loa* since this filarial worm lacks *Wolbachia* endosymbionts. Therefore doxycycline can be used for the treatment of lymphatic filariasis in co-endemic areas without the risk of severe adverse reactions (Bockarie *et al.* 2010). Anti-wolbachial treatment also decreases immune reactions that appear when *Wolbachia* are released after the death of filaria, thus having a beneficial effect on treated patients (Pfarr *et al.* 2009; Tamarozzi *et al.* 2011). Today the antibiotics doxycycline and rifampicin are successfully used for the treatment of filariasis and a good macrofilariacidal (> 70%) effect has been observed (Taylor *et al.* 2010) . However, doxycycline cannot be used for the treatment of children or pregnant women and both antibiotics have to be administered daily for a period of several weeks.

Therefore these drugs are not qualified for the use in mass drug administration programs, only for individual physician monitored treatment. The development of novel substances that are suitable for mass drug administration and that target the *Wolbachia* endosymbionts, thus resulting in the death of adult worms, is of urgent need.

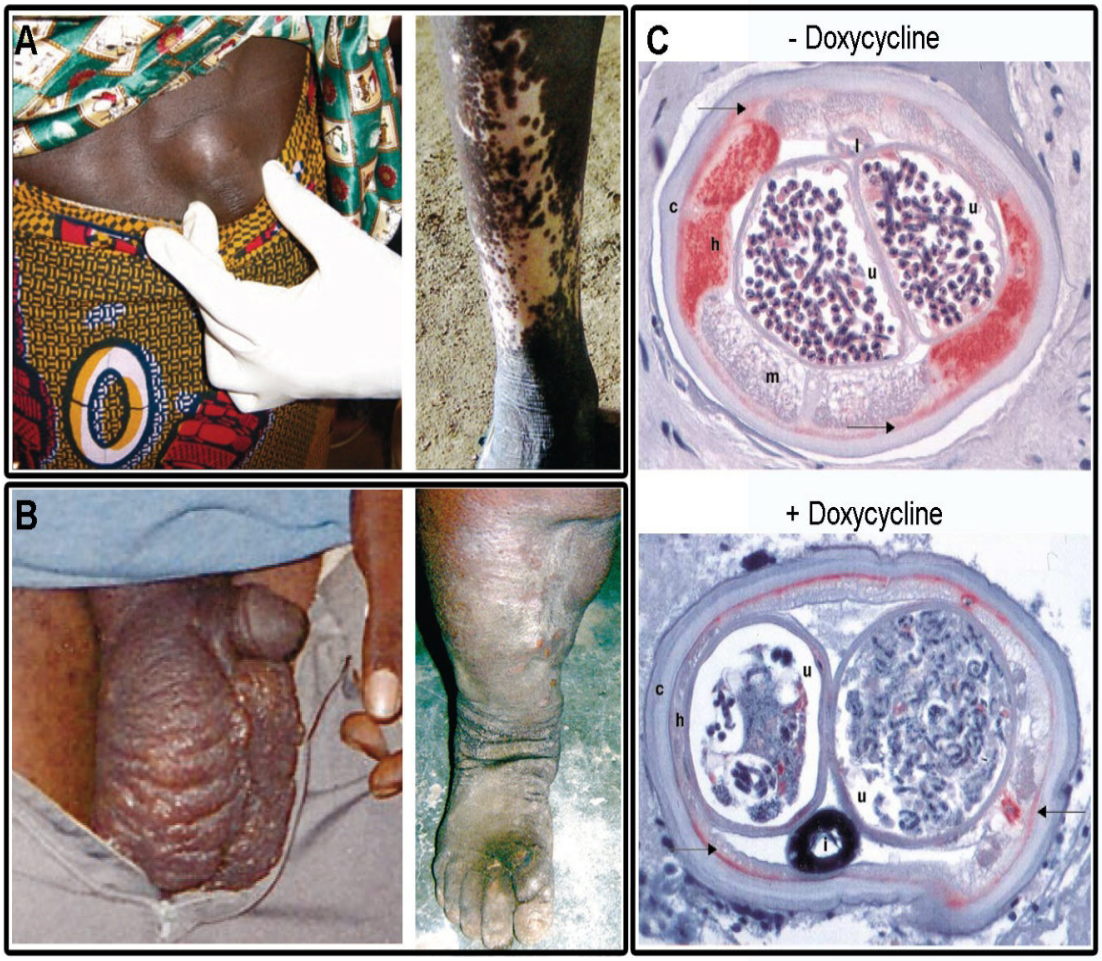


Figure 1.5: Severe pathologies of filariasis and effects of the depletion of *Wolbachia* on the worm (Hoerauf *et al.* 2001; Taylor *et al.* 2010). (A): Worms of the species *Onchocerca volvulus* causing onchocerciasis reside in subcutaneous skin nodules (left panel). Released microfilariae migrate through the skin whereupon they can cause severe skin disease (right panel). **(B):** In lymphatic filariasis the dead adult worms inside the lymphatic vessels can result in hydrocele (left panel) or lymphedema (right panel). **(C):** Endosymbiotic *Wolbachia* are essential for the survival of filarial worms. In untreated worms *Wolbachia* can be found mainly in the hypodermal chords, as well as in embryos of female worms (upper panel, *Wolbachia* stained in red). After treatment with doxycycline *Wolbachia* are depleted and embryos are degenerated (lower panel). h: hypodermal chords, c: cuticle, m: musculature, i: intestine, u: uterus epithelium, arrows: less intense staining possibly of nematode mitochondrial hsp-60.

1.2.2 Lipid II metabolism in *Wolbachia*

In *Wolbachia* that possess only limited metabolic pathways it is hypothesized that retained genes are crucial for survival (Foster *et al.* 2005). The characterization of retained pathways that are essential for *Wolbachia* is needed to better understand *Wolbachia*

biology and to identify novel potential drug targets. As for other intracellular bacteria, *Wolbachia* are protected from osmotic stress. Thus, they do not need a stabilizing cell wall and peptidoglycan has never been detected. But surprisingly they harbor many genes for lipid II metabolism as discovered by genome annotation (Foster *et al.* 2005). Among these are all enzymes necessary to synthesize lipid II from UDP-GlcNAc (Figure 1.6). However, the composition of the pentapeptide is unknown. Interestingly, of the few amino acid biosynthesis pathway retained in *Wolbachia* (Dunning Hotopp *et al.* 2006) one is necessary to synthesize *meso*-DAP, an amino acid that is commonly found in the peptide of lipid II in Gram-negative bacteria (Vollmer, W. *et al.* 2008b). *Wolbachia* also possess the gene for the D-alanine-D-alanine ligase (DDL) required to synthesize the D-alanine dipeptide that is normally linked to the UDP-MurNac-tripeptide by MurF, yet the genes for the L-alanine racemase *alr* and L-glutamate racemase *murI* are missing. Thus, whether *Wolbachia* are able to generate D-amino acids or use the L-form instead is still a controversial question.

A striking feature of *Wolbachia* is that glycosyltransferases are missing from their genomes. Thus, the bacteria cannot generate the long glycan strands which commonly build the backbone of peptidoglycan. Although the genes necessary to synthesize lipid II can be found it is unclear if they possess any enzyme that is able to replace the glycosyltransferases in catalyzing the cleavage of the link between lipid II and the membrane anchor. They also lack almost all peptidoglycan degrading and recycling enzymes except the amidase AmiD, although it can only be found in the *Wolbachia pipientis* endosymbiont of *Drosophila melanogaster* (*w*Mel) and *Culex quinquefaciatus Pel* (*w*Pa), not in the filarial *Wolbachia* endosymbiont of *B. malayi* (*w*Bm). However, each strain contains a carboxypeptidase and one or two transpeptidases of the PBP protein family. For this reason it was supposed that *Wolbachia* might harbor an unusual peptidoglycan-like molecule that lacks glycan strands and is instead built of lipid II molecules that are linked by peptide cross-bridges (Foster *et al.* 2005). Another hypothesis is that the lipid II synthesis pathway might be involved in the coordinated function of the divisome and is therefore conserved in *Wolbachia* (Henrichfreise *et al.* 2009). This hypothesis was supported by the observations that in *Bacillus spp.* cell division was blocked due to the depletion of lipid II by antibiotics (Hasper *et al.* 2006; Hyde *et al.*

2006). Notably, similar results were found in chloroplasts of moss subsequent to the disruption of MurE, one of the lipid II synthesizing enzymes (Machida *et al.* 2006).

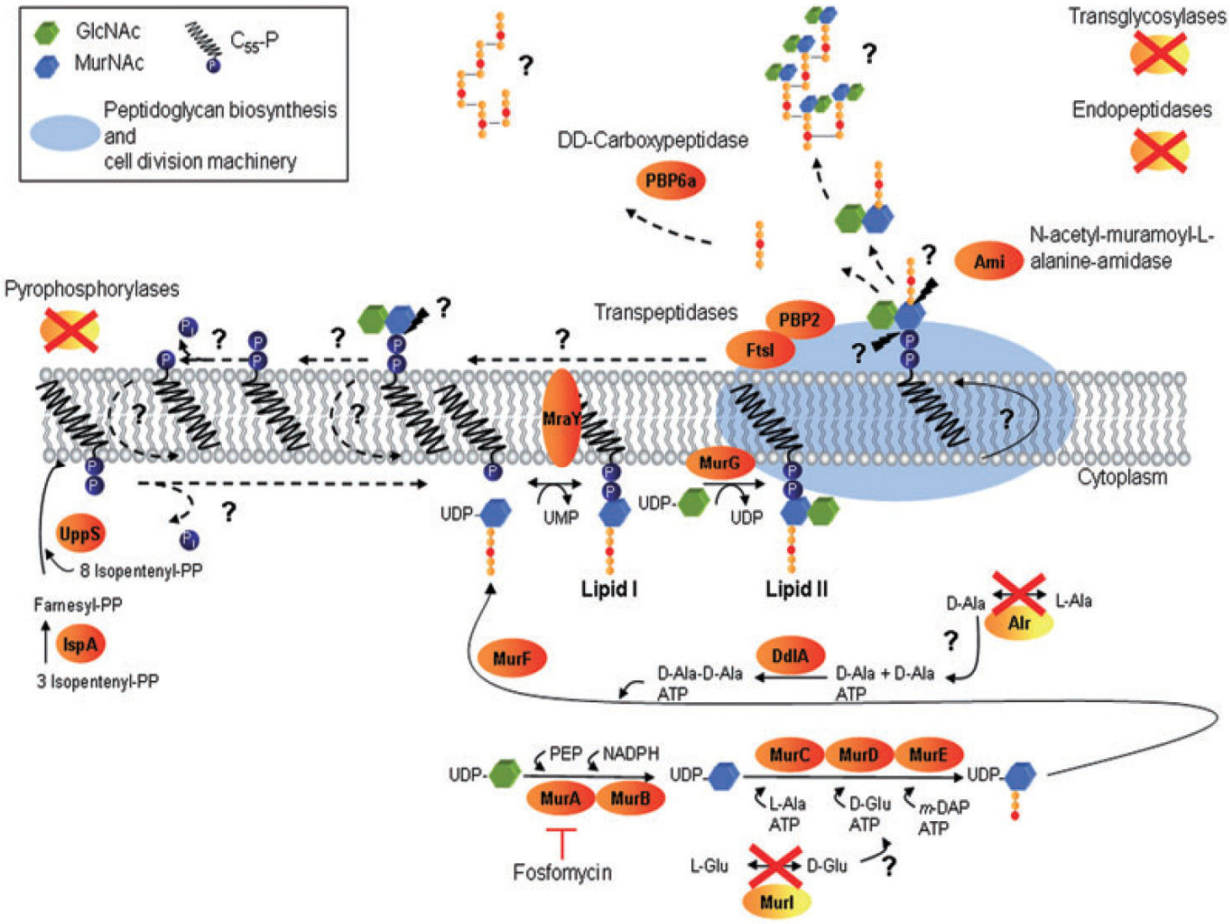


Figure 1.6: Proposed model of lipid II metabolism in *Wolbachia* (Henrichfreise *et al.* 2009). Lipid II biosynthesis takes place in the cytoplasm catalyzed by the enzymes MurA to MurF, MraY and MurG but racemases necessary to generate D-amino acids are missing. Lipid II is then flipped into the periplasm where it might be cross-linked by the action of carboxypeptidases and transpeptidases. However, glycosyltransferases, endopeptidases and almost all peptidoglycan recycling enzymes are missing. Therefore *Wolbachia* cannot form long glycan chains and, although they possess the genes needed to synthesize undecaprenyl phosphate, no alternative enzyme able to cleave the sugar moiety of lipid II off the lipid anchor is known. Those genes that are not found in the *Wolbachia* genome are depicted with red crosses. Question marks and dashed lines indicate yet unidentified metabolic steps.

Until today not much is known about the functionality of lipid II metabolism in *Wolbachia*. It has been be demonstrated that *in vitro* the recombinant *Wolbachia* enzymes MraY and MurG are able to synthesize lipid I and lipid II, respectively (Henrichfreise *et al.* 2009). Moreover, lipid II could be synthesized from the precursor UDP-MurNAc-pentapeptide by isolated *Wolbachia* membranes, thus confirming the activity of MraY and MurG *ex vivo*. Using a C6/36 insect cell line infected with *Wolbachia*

pipientis strain *A. albopictus B* (*w*Alb B) it could be furthermore shown that the treatment of the cells with the antibiotic fosfomycin, a specific inhibitor of MurA, led to the depletion of *Wolbachia* from the insect cells. These results strongly indicate the functionality of the lipid II biosynthesis pathway in *Wolbachia*, but the exact tertiary structure of lipid II is still unidentified. It is unknown whether *Wolbachia* are able to generate a peptidoglycan-like molecule that surrounds their cells and the role of lipid II or a further processed molecule in the environmentally protected endobacteria is still to be elucidated.

1.3 Objectives

Based on earlier research evidence was given that the cell wall precursor lipid II might participate in the cell division of *Wolbachia* endobacteria. The characterization of the lipid II biosynthesis in *Wolbachia* might not only discover the role of lipid II in these cell wall-less bacteria, but might reveal new potential targets for the development of novel antibiotics. Part of this PhD project was to elucidate the role of lipid II in the biology of *Wolbachia* and to verify the hypothesis of an essential function in cell division. Furthermore the structure of lipid II should be investigated in *Wolbachia* that lack several genes necessary to form the characteristic composition of the molecule.

The investigation of *Wolbachia* endobacteria is extremely challenging since *in vitro* culturing of the bacteria is only successful inside insect cell lines. An extracellular *Wolbachia* culture system would provide a powerful tool for the elucidation of *Wolbachia* biology as well as for drug screenings. Therefore, in a second part of this PhD project, requirements necessary for *Wolbachia* replication outside their host cell were characterized to gain new insights needed for the establishment of a *Wolbachia* host cell-free culture system.

2 Materials and Methods

2.1 Materials

2.1.1 Bacterial strains

- *Aedes albopictus* cell line C6/36 (Turner *et al.* 2006)
- *Aedes albopictus* cell line C6/36 infected with *Wolbachia pipientis* strain *Aedes albopictus B (w*Alb B) (Turner *et al.* 2006)
- One Shot TOP10® chemically competent *E. coli* (Invitrogen, Darmstadt, Germany)
- *E. coli* BL21 (Novagen, Darmstadt, Germany)

2.1.2 Antibodies

- Rabbit *Wolbachia* FtsZ anti-serum (Sullivan Laboratory, University of California, USA (Landmann *et al.* 2012))
- Rabbit *w*PAL anti-serum (Taylor Laboratory, University of Liverpool, UK (Turner *et al.* 2009))
- Goat anti-rabbit alexa fluor 488 conjugate secondary antibody (Invitrogen, Darmstadt, Germany)
- Goat anti-rabbit gold conjugate (10nm) secondary antibody (BBInternational, Cardiff, UK)

2.1.3 Chemicals, buffers, gels and media

Chemicals:

- 1-Bromo-3-chloro-2-propanol (Sigma-Aldrich, Steinheim, Germany)
- 50bp DNA ladder (New England BioLabs, Frankfurt, Germany)
- Bovine serum albumin; BSA (Fraction V, Fisher, Schwerte, Germany)
- 4′,6-Diamidin-2-phenylindol; DAPI (Sigma-Aldrich, Steinheim, Germany)
- Fosfomycin (Infectofos, Infectopharm Arzneimittel, Heppenheim, Germany)
- Glutaraldehyde (Fluka, Seelze, Germany)

- Glycine (Merck, Darmstadt, Germany)
- Goat serum (PAA Laboratories, Cölbe, Germany)
- Isopropyl β-D-1-thiogalactopyranoside, IPTG (Sigma-Aldrich, Steinheim, Germany)
- Lead citrate (Serva, Heidelberg, Germany)
- LRWhite (Agar Scientific, Essex, UK)
- N,N,N',N' - Tetramethylethylendiamin, 1,2 - Bis(dimethylamino) – ethan; TEMED (Roth, Karlsruhe, Germany)
- Ni-NTA agarose (Qiagen, Hilden, Germany)
- Novex® Sharp protein standard prestained (Invitrogen, Darmstadt, Germany)
- Osmium tetroxide (Sigma-Aldrich, Steinheim, Germany)
- Paraformaldehyde (Merck, Darmstadt, Germany)
- Propylene oxide (Electron Microscopy Sciences, Hatfield, PA, USA)
- RedSafe™ DNA stain for agarose gels (Chembio, Hertfordshire, UK)
- Rotiphorese® Gel 30, Acrylamid-, Bisacrylamid 37.5:1 stock (Roth, Karlsruhe, Germany)
- Sodium dodecyl sulfate; SDS (Roth, Karlsruhe, Germany)
- Sodium azide (Sigma-Aldrich, Steinheim, Germany)
- Sodium cacodylate trihydrate (Sigma-Aldrich, Steinheim, Germany)
- Sodium hydroxide discs (Merck, Darmstadt, Germany)
- SYBR Green® (Fermentas, St. Leon-Rot, Germany)
- Tricine (Sigma-Aldrich, Steinheim, Germany)
- Trisma Base® (Sigma-Aldrich, Steinheim, Germany)
- Triton X-100 (Sigma-Aldrich, Steinheim, Germany)
- TRIzol® Reagent (Invitrogen, Darmstadt, Germany)
- Uranylacetate (Leica, Wetzlar, Germany)
- Vectashield® Mounting Medium (Vector Laboratories, Burlingame, CA, USA)

Buffers and gels:

- 10x Phosphate Buffered Saline, PBS (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7,4)
- PBST (PBS, 2 % Bovine Serum Albumin (w/v), 0.1 % Triton X-100)
- 10 x TBE buffer (890 mM tris base, 890 mM boric acid, 20mM EDTA, pH 8)

- Lysis buffer for protein purification (100mM Tris, 300 mM NaCl, 20 mM imidazole, pH 8)
- Wash buffer for protein purification (100 mM Tris, 300 mM NaCl, 20 mM imidazole, pH 8)
- Elution buffer for protein purification (100 mM Tris, 300 mM NaCl, 250 mM imidazole, 10 % glycerol, pH 8)
- Wash buffer for immunogold staining (TBS pH 8.2, 1 % goat serum, 1 % BSA, 0.1 % sodium azide)
- SDS-PAGE running buffer (100 mM Tris Base, 100mM Tricine, 35mM SDS)
- 5x SDS-PAGE loading buffer (25 mM TrisHCl pH 6.8, 25 % glycerol, 7.5 % SDS, 0.04 % bromphenolblue (w/v), 12.5 % b-mercaptoethanol)
- SDS-PAGE 12.5 % separating gel (375 mM Tris, 0.1 % SDS, pH 8.8, 12.5 % acrylamide, 0.05 % ammonium persulfate, 0.05 % TEMED)
- SDS-PAGE stacking gel buffer (125 mM Tris, 0.1 % SDS, pH 6.8, 4.5 % acrylamide 0.03 % ammonium persulfate, 0.2 % TEMED)

Media:

- L15 Leibovitz medium (Invitrogen, Darmstadt, Germany) + 5 % FCS (PAA Laboratories, Cölbe, Germany), 1 % MEM nonessential amino acids (Sigma-Aldrich, Steinheim, Germany), 2 % tryptose phosphate broth (Sigma-Aldrich, Steinheim, Germany) and 1 % penicillin/streptomycin (PAA Laboratories, Cölbe, Germany)
- Super optimal broth with catabolic repressor medium, SOC medium (Invitrogen, Darmstadt, Germany)
- Lysogeny broth medium, LB medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl (w/v))
- Lysogeny broth agar, LB agar (LB medium, 1.5 % agar (w/v))

2.1.4 Enzymes and commercial kits

Enzymes:

- Alanine racemase of *Bacillus stearothermophilus* (Sigma-Aldrich, Steinheim, Germany)
- Benzonase® (Novagen, Darmstadt, Germany)

- D-amino acid oxidase from porcine kidney, DAAO (Sigma-Aldrich, Steinheim, Germany)
- Lysozyme (Novagen, Darmstadt, Germany)

Kits:

- BrdU cell proliferation assay Kit (Calbiochem, Darmstadt, Germany)
- DNA-*free*™ Kit (Applied Biosystems, Darmstadt, Germany)
- EMbed 812 Kit (Electron Microscopy Sciences, Hatfield, PA, USA)
- HotStarTaq® DNA Polymerase Kit (Qiagen, Hilden, Germany)
- Omniscript® RT Kit (Qiagen, Hilden, Germany)
- QIAamp DNA Mini Kit (Qiagen, Hilden, Germany)
- QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany)
- TOPO® TA Cloning Kit (Qiagen, Hilden, Germany)

2.1.5 Primers, vectors and templates

Primer:

- Random decamer primers (Ambion, Darmstadt, Germany)
- T7 promotor sequencing primer (5`-TAATACGACTCACTATAGGG-3', Invitrogen Darmstadt, Germany)
- 16S rRNA primer for real-time PCR (forward primer 5' – TTGCTATTAGATGAGCCTATATTAG-3', reverse primer 5'-GTGTGGCTGATCATCCTCT-3', Biolegio, Nijmegen, Netherlands)
- Sequences of specific PCR-primers used for reverse transcriptase PCR are listed in Table 2.1

Table 2.1: Sequences and characteristics of PCR-primer pairs

Gene	Locus tag	Forward primer [5´- 3´]	Reverse primer [5´- 3´]	Annealing temperature [°C]	Product size [bp]
murA	WPa_0760	CGCATAGAGGCCGGTACTTA	ATGGATTTGTTGCAACGTGA	51.5	202
murB	WPa_0473	GCAAGGCATGGAAACTGATT	CCAAAACCCTTATCTCCCACT	66	201
murC like	WPa_0207	CCCTGGCTGCAATATCAGTT	TTACGAACACGAGCAAAACG	60	247
murD	WPa_0533	GGTAGCAAGATTGCCGTGAT	AGCCCTGGAAAGGATTTGAT	56	281
murE	WPa_0137	GATGAAGATCCGGGGAAAAT	GCGTTGGATGAAGGAGAGAG	56	257
murF	WPa_0848	TGGCACTACTTGGCGATATG	TTCCTGCACTACAAGGTCCA	50	275
rodA	WPa_1016	TTGTAAAAGTGGGCTTAATACTTGC	TTAAATGAGGTCTTTCCATTATTGC	62,6	216
amiD	WPa_0833	AATGCGTAAGCCTGATCCAC	TGATTGATAACCCACGCAAA	59	294
dapE	WPa_0691	ATACTGGTAATAAATATTTTCAGCCTTCAC	CTCTGCATAGACAGTTTATAATCGTTAGTT	60	205
dapF	WPa_0959	ATGGTAAGTAACCTAACAGACAAGATCC	CATTGTAGATATGCATAAAGCAGTTTG	55	196
metC	WPa_0138	GGCTGGAAAAACATCCAAAA	ACTGTCACATCCTCCCCAAG	62	209
glyA	WPa_0836	GGTTCTCAGGCAAATCAAGC	ACGAAAGCGCTCGAAATCTA	62	273
ddl	WPa_0403	TAATGGAAAAGATGATCATAGAAGAATACA	TCCAAGGTTATTCTATATATGTTGTTAGGA	55	198
pbp2	WPa_0573	GTTATAGGACAAGGGTATTTACTTACAACG	GCCTAGTCCTTTTCTATAGTTAGAATTCAC	62	210
pbp6a	WPa_1357	GAATAGAGGAAGCAAAAAGACTAATACAAT	TACCATATCTTTATACTCAATACGCACTTT	60	212

Vectors:

- pCR®2.1-TOPO® cloning vector (Invitrogen, Darmstadt, Germany)
- pET-21b(+) expression vector (Novagen, Darmstadt, Germany) containing *metC* of *Wolbachia* endosymbiont of *B. malayi* (NheI and XhoI restriction sites) (Jülicher 2012)

Templates:

- Genomic DNA from an *A. albopictus* cell line C6/36 infected with *w*Alb B
- Genomic DNA prepared from insect cell-free *Wolbachia* culture
- cDNA from an *A. albopictus* cell line C6/36 infected with *w*Alb B

2.1.6 Equipment and software

Equipment:

- 96-well and 24-cell culture well plates, clear, flat bottom, with lid (Greiner, Frickenhausen, Germany)
- Bench-top centrifuges: 5417R (Eppendorf, Hamburg, Germany) and Mikro 200 (Hettich, Tuttlingen, Germany)
- 3mm borosilicate glass beads (Sigma-Aldrich, Steinheim, Germany)
- CELLSTAR® standard cell culture flasks 25 cm^2 and 75 cm^2 (Greiner, Frickenhausen, Germany)
- Cell density meter model 40 (Fisher scientific, Schwerte, Germany)
- Corning cell lifter (Sigma-Aldrich, Steinheim, Germany)
- Culture slides, 8-well (BD Falcon, Heidelberg, Germany)
- Diamont knive 2.5 mm, 45 °C (Diatome, Biel, Switzerland)
- Digital camera KY-F75U (JVC, Wayne, NJ, USA)
- Dynal Sample Mixer (Invitrogen, Darmstadt, Germany)
- Electron image film (Kodak, Science Services, Munich, Germany)
- DM-RD fluorescence microscope (Leica, Wetzlar, Germany)
- Nanovue™ (GE Healthcare, Munich, Germany)
- Neubauer counting chamber improved (Laboroptik, Bad Homburg, Germany)
- Polypropylene column (Qiagen, Hilden, Germany)
- QIAcube robotic workstation (Qiagen, Hilden, Germany)

- Rotorgene 6000 (Corbett Life Sciences, Sydney, Australia)
- Mini-PROTEAN Tetra Electrophoresis System for SDS-PAGE (Bio-Rad, Munich, Germany)
- Shaker Certomat® (Sartorius, Goettingen, Germany) and KS 250 basic (IKA Labortechnik, Staufen, Germany)
- Sonicator Sonoplus HD 2200 (Bandelin, Berlin, Germany)
- Sorvall Evolution EC Superspeed Centrifuge (Fisher scientific, Schwerte, Germany)
- Spectramax 340PC (Molecular Devices, Biberach an der Riss, Germany)
- TEM900 electron microscope (Zeiss, Goettingen, Germany)
- Thermocycler iCycler (Bio-Rad, Munich, Germany) and Cycler Primus (MWG-Biotech, Ebersberg, Germany)
- Ultracut R ultra-microtome (Leica, Wetzlar, Germany)

Software:

- Photoshop® elements version 3 (Adobe®, Munich, Germany)
- Bioedit Biological Sequence Alignment Editor (Ibis Biosciences, CA, USA)
- Diskus microscope software ver 4.60.296-#391 (Carl Hilgers Technisches Büro, Königswinter, Germany)
- GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA)
- SoftMax Pro 5.4.1 (Molecular Devices, Biberach an der Riss, Germany)

2.2 Methods

2.2.1 Cell Biology Methods

2.2.1.1 Insect cell culture

Wolbachia endosymbionts of filarial nematodes cannot be successfully maintained in cell culture. For this reason cell culture experiments were performed with *A. albopictus* C6/36 insect cell lines uninfected or infected with *w*Alb B. Insect cells were cultured as previously described (Turner *et al.* 2006; Henrichfreise *et al.* 2009). Infected and uninfected C6/36 cells were incubated in plug-sealed 75 cm^2 flasks at 26 °C in L15

Leibovitz's medium supplemented with 5 % FCS, 1 % nonessential amino acids, 2 % tryptose phosphate broth and 1 % penicillin/streptomycin and were passaged every 7 days.

2.2.1.2 Isolation of *Wolbachia* from insect cells

Wolbachia were purified from infected C6/36 cells either as described by *Rasgon et al.* (Rasgon et al., 2006) or by an abbreviated protocol. For *Wolbachia* purification following the procedure of Rasgon *et al.*, insect cells were grown in a plug-sealed 75 cm^2 culture flask to ~90 % confluence and detached by scraping in 10 ml L15 Leibowitz medium. Cells were lysed by vortexing with 100 sterile 3mm borosilicate glass beads for 5 min. Cell debris was removed by centrifugation at 2,500 g for 10 min at 4 °C and the supernatant was filtered through a 5 µm syringe filter. *Wolbachia* of 1.3 ml fractions were pelleted by centrifugation at 18,400 g for 5 min at 4 °C on a 250 mM sucrose cushion of 200 µl volume. Pellets were suspended in 10 ml L15 Leibowitz's medium and filtered through a 1.2 µm syringe filter. The number of *Wolbachia* was determined by quantitative real-time PCR (qPCR) using DNA extracted from 200 µl *Wolbachia* suspensions as template (section 2.2.2.1 and 2.2.2.4). Since the *Wolbachia* 16S rRNA gene is a single copy gene, each gene copy equaled one *Wolbachia* cell.

For the abbreviated purification protocol, *Wolbachia* were isolated as described above until the filtration step through the 5 µm syringe filter. The amount of *Wolbachia* in this cell lysate was also determined by qPCR.

2.2.1.3 Cell-free *Wolbachia* culture

To investigate the effect of insect cell lysate on isolated *Wolbachia* the bacteria were purified from insect cells either using the procedure published by Rasgon *et al.* or by an abbreviated protocol section 2.2.1.2). 1 ml isolated *Wolbachia* with or without insect cell lysate was mixed with 4 ml Leibowitz's medium and incubated in plug-sealed 25 cm^2 cell culture flasks for 7 days at 26 °C. 200 µl samples were removed at days 0, 1, 3, 5 and 7, DNA was extracted (section 2.2.2.1) and the number of *Wolbachia* was calculated by qPCR (section 2.2.2.4).

For insect cell lysate titration assays, a concentrated *Wolbachia* suspension with a concentration of 10^4 – 10^5 16S rRNA gene copies/µl was generated by the abbreviated protocol. Insect cell lysate was also prepared by the abbreviated protocol using

uninfected C6/36 cells. The amount of uninfected insect cells was calculated previous to cell lysis using a Neubauer counting chamber. Isolated *Wolbachia* were added to insect cell lysate to a final concentration of 0.5 – 1 x 10^3 16S rRNA gene copies/µl and 200 µl reactions were incubated in 96 well plates for 12 days at 26 °C. Triplicate wells were harvested at days 0, 3, 6, 9 and 12 and the amount of *Wolbachia* was calculated by qPCR.
For *Wolbachia* cell number titration assays, the bacteria were isolated according to the abbreviated protocol. Decreasing amounts of *Wolbachia* were diluted in insect cell lysate prepared from 0.95 x 10^6 uninfected insect cells and 200 µl reactions were incubated in 96 well plates for 12 days at 26 °C. Triplicates were harvested by pipetting repeatedly at days 0, 3, 6, 9 and 12 and the amount of *Wolbachia* was calculated by qPCR.

For insect cell lysate fractionation assays, insect cell lysate from uninfected insect cells and a *Wolbachia* suspension were prepared according to the abbreviated protocol. The amount of uninfected insect cells was determined using a Neubauer counting chamber prior to cell lysis. The insect cell lysate was fractionated by centrifugation at 4 °C at 20,000 g for 30 min or at 100,000 g for 1 h, respectively. The supernatants containing microsomes and plasma membranes or the soluble cytoplasmic content, respectively, were retained. Since ultracentrifugation could not be performed under sterile conditions the supernatant obtained after centrifugation at 100,000 g for 1h was sterile filtered and the pellet discarded. The pellet obtained after centrifugation at 20,000 g for 30 min containing nuclear debris and large organelles was dissolved in the same volume of L15 Leibowitz's medium. Fractions were used for the preparation of cell-free *Wolbachia* cultures with a concentration of 0.5 – 1 x 10^3 16S rRNA gene copies/µl. The volume of fraction added to the culture was equivalent to a concentration of 0.95 x 10^6 insect cells/ml. Triplicates were harvested on days 0, 3, 6, 9 and 12 and the amount of *Wolbachia* was calculated by qPCR.

For *Wolbachia* in cell-free culture with and without FCS, *Wolbachia* were purified according to the abbreviated procedure. Two different insect cell lysates were prepared according to the abbreviated method, one generated in cell culture medium containing 5 % FCS and one in cell culture medium lacking FCS. Previous to the preparation of insect cell lysate without FCS, the insect cells were washed once in cell culture medium lacking FCS. Both lysates were centrifuged at 20,000 g for 30 min at 4 °C and the supernatants were retained (membrane fraction of lysate). *Wolbachia* cultures containing membrane

fractions with and without FCS were incubated at 26 °C for 12 days. A negative control containing *Wolbachia* incubated in cell culture medium was added. The initial *Wolbachia* concentration was between 2 and 4 x 10^3 16S rRNA gene copies/µl and the final concentration of insect cells in the membrane fraction was equivalent to 0.95 x 10^6 insect cells/ml as calculated from the amount of cells counted prior to lysis. Growth was monitored on days 3, 6, 9 and 12 by qPCR.

2.2.1.4 Infection of C6/36 insect cells with *Wolbachia*

The ability of *Wolbachia* incubated in cell-free culture to infect new C6/36 insect cells was determined by infection of uninfected C6/36 insect cells. A cell-free *Wolbachia* culture with a concentration of 10^3 16S rRNA gene copies/µl supplemented with the membrane fraction obtained from cell lysate of uninfected insect cells was prepared as described in sections 2.2.1.2 and 2.2.1.3. *Wolbachia* were incubated at 26 °C for 12 days. On day 9 uninfected insect cells were seeded in a 24-well plate with a concentration of 10^5 insect cells/well. Infection was performed in triplicate. On day 12 medium was removed from uninfected insect cells and 750 µl from the insect cell-free *Wolbachia* culture was added. As a negative control *Wolbachia* culture was heated at 95 °C for 10 min, cooled down and subsequently applied to uninfected insect cells. The uninfected insect cells covered with cell-free *Wolbachia* culture were centrifuged at 2,000 g for 1 h at 15 °C and subsequently incubated at 26 °C over night. On the next day, cells were harvested and transferred into a 6-well plate containing 1.5 ml cell culture medium supplemented with 10 % FCS and incubated at 26 °C. After 6 days the cells were harvested in 2 ml freshly added cell culture medium, transferred to culture slides (300 µl suspension/well) and incubated at 26 °C for 1 day. To verify the success of *Wolbachia*-infection the infected insect cells grown on culture slides were examined by immunofluorescence microscopy (section 2.2.4.1).

2.2.2 Molecular Biology Methods

2.2.2.1 DNA extraction

Genomic and plasmid DNA were extracted using the QIAamp DNA Mini Kit and QIAprep Spin Miniprep Kit, respectively, according to the manufacturer's protocol. DNA of multiple samples was extracted using a QIAcube robotic workstation.

2.2.2.2 RNA extraction

Wolbachia of filarial nematodes cannot be successfully maintained in cell culture, for this reason total RNA was extracted from *w*Alb B infected and uninfected *A. albopictus* C6/36 cell lines. Insect cells were grown in plug-sealed 75 cm^2 culture flasks to ~90 % confluence. RNA isolation followed a standard Trizol reagent protocol. For this, cells were harvested at 2,500 g for 8 min at 4 °C and pellets were suspended in 1 ml Trizol reagent and mixed vigorously with 0.1 ml 1-Bromo-3-chloro-2-propanol for 15 seconds. The mixture was incubated for 10 min at room temperature and centrifuged afterwards for 15 min at 12,000 g at 4 °C. The upper aqueous phase containing the nucleic acids was retained and the nucleic acids were precipitated by the addition of 0.5 ml isopropanol and by incubation at -20 °C for 10 min. After a centrifugation step at 12,000 g for 15 min at 4 °C the supernatant was discarded and the pellet was washed in 1 ml 75 % ethanol. The ethanol was removed by centrifugation at 7,500 g for 4 min at 4 °C and the pellets were dried at 37 °C and subsequently suspended in nuclease free water.

To remove contaminating DNA the extracted RNA was treated with the DNA-*free* Kit according to the manufacturer's protocol. The yield of total RNA was measured using a Nanovue and aliquots were stored at -80 °C.

2.2.2.3 Two-step reverse transcriptase PCR

The transcription of specific genes was analyzed by a two-step reverse transcriptase PCR (RT-PCR). Reverse transcription was performed using the Omniscript Reverse Transcriptase Kit according to the manufacturer's protocol. 75 ng/µl RNA was reverse transcribed to cDNA using 10 µM random decamer primers. 200 – 300 bp fragments of genes involved in peptidoglycan metabolism were amplified with gene specific primers (Table 2.1) using cDNA as template and genomic DNA as a control. PCR was carried out in 20 µl reactions using 0.5 U Hot Star Taq polymerase, 3 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM primer, 1 x PCR buffer and 2 µl template. The PCR conditions included a heat activation step for 15 min at 95 °C following 35 cycles of 30 sec at 94 °C, 30 sec at a primer dependent temperature (Table 2.1), 1 min at 72 °C and a final extension step for 10 min at 72 °C. PCR products were analyzed on 2 % agarose gels, cloned into a TOPO vector using TOPO® TA cloning kit according to the manufracturer´s protocol and sequenced at Seqlab sequencing laboratories (Goettingen, Germany) using a T7 promotor primer. The

analysis of the genes *murA* - *murF*, *rodA*, *metC* and *glyA* was performed as part of a bachelor thesis supervised during this PhD project (Jülicher 2012).

2.2.2.4 Quantitative real-time PCR

Wolbachia cell numbers were calculated by quantification of 16S rRNA gene copies using qPCR. Performance of qPCR was supported by technical assistance. A qPCR reaction contained 1 x HotStar Taq polymerase buffer, 3 mM $MgCl_2$, 200 µM dNTPs, 0.2 µl SYBR Green (1:1000 dilution in DMSO), 0.5 µM 16S rRNA gene specific forward and reverse primer, respectively, and 0.5 U HotStar Taq polymerase. 2 µl DNA extracted from 200 µl *Wolbachia* suspension was used as template. qPCR conditions included a heat activation step at 95 °C for 15 min followed by 45 cycles of 95 °C for 10 sec, 55 °C for 15 sec and 72 °C for 20 sec. Melt curve analysis showed a specific peak for all positive samples. Data were analyzed using Rotorgene 6000 software version 1.7.

2.2.2.5 BrdU cell proliferation assay

Besides qPCR, replication of insect cell-free *Wolbachia* was detected using the BrdU cell proliferation assay kit. In this assay, the thymidine analog bromodeoxyuridine (BrdU) is used, which is incorporated into newly synthesized DNA during replication. Subsequently, incorporation is detected by an ELISA based method. For detection of *Wolbachia* replication BrdU was supplemented to cell-free *Wolbachia* culture to a final dilution of 1:10,000. A negative control of cell-free *Wolbachia* culture without BrdU was included in the experiment. Reactions of 0.2 to 2 ml culture were incubated in 24-well plates or 25 cm^2 for 12 days and triplicate samples were harvested every 3 days by centrifugation at 18,400 g for 5 min at 4 °C. Pelleted *Wolbachia* were incubated in 200 µl fixative/denaturing solution for 30 min at room temperature to fix the cells and to denature DNA necessary for proper antibody binding. Subsequent to centrifugation at 18,400 g for 5 min at room temperature, the supernatant was discarded and the pellet was incubated with 40 µl a BrdU monoclonal antibody diluted 1:100 in antibody dilution buffer for 1 h at room temperature. Unbound antibody was removed by 3 washing steps in 300 µl wash buffer accompanied by repeated centrifugations at 18,400 g for 5 min. In the next step 40 µl of peroxidase-conjugated secondary antibody was added in a 1:1000 dilution and incubated for 30 min at room temperature. After 3 washing steps with wash

buffer and 1 washing step with demineralised water accompanied by centrifugation at 18,400 g for 5 min, 100 µl substrate solution containing the peroxidase substrate tetramethylbenzidine was added and incubated for 15 min in the dark. The reaction was stopped by the addition of 100 µl stop solution. Samples were transferred to a 96-well plate and absorbance was detected immediately in an ELISA plate reader at 450 nm wavelength.

2.2.3 Protein Biochemistry Methods

2.2.3.1 Expression and purification of *Wolbachia* MetC

Cloning of the *w*Bm *met*C gene and optimization of expression and purification conditions were carried out as part of a bachelor thesis supervised during this PhD project (Jülicher 2012).

For protein expression *E. coli* BL21 cells were grown in 1 L cultures at 37 °C until OD_{600} of 0.4. The *E. coli* cells were further grown at room temperature until OD_{600} of 0.6 and protein expression was induced by addition of 0.25 mM IPTG leading to an overproduction of recombinant *w*Bm MetC containing a C-terminal His_6-tag. The *E. coli* cells were harvested after 2 h at 4,650 g for 10 min at 4 °C and stored at -20 °C. To purify the recombinant protein the pellets were suspended in 20 ml lysis buffer and incubated on ice with 200 µg/ml lysozyme and 10 U/ml Benzonase for 30 min. The cells were disrupted by sonification for 6 x 10 sec at 60 % power on ice and subsequently centrifuged at 20,000 g for 15 min at 4 °C. The supernatant was mixed with 0.1 % (v/v) Ni-NTA agarose for 2 h at 4 °C and afterwards loaded onto a polypropylene column. After three washing steps with 8 ml wash buffer the His_6-tagged protein was eluted in 8 x 500 µl elution buffer, the amount of protein was determined by Bradford assay according to the manufacturer's protocol and aliquots were stored at -80 °C.

2.2.3.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To analyze the purity of recombinant protein, the protein of the elution fractions were separated by molecular weight using SDS-PAGE (Laemmli 1970). Protein samples were diluted until a final concentration of 0.2 µg/µl protein, mixed with 5 x SDS-PAGE loading buffer to a 1 x dilution and denatured at 95 °C for 5 min. 20 µl of each sample were

loaded onto a 1 mm 4.5 % stacking gel and separated in a 12.5 % separating gel by applying in running buffer to an electric field for 45 min at 180 V. Protein was stained with Coomassie brilliant blue for 20 min and destained in 30 % methanol.

2.2.3.3 *In vitro* activity assay of recombinant MetC

L-alanine racemase activity was analyzed in a coupled enzymatic assay using a modified method by Francois *et al.* (Francois *et al.* 2007). The optimal recombinant protein concentration and assay buffer was investigated as part of a bachelor thesis (Jülicher 2012).

To analyze L-alanine racemase activity 0.4 µg/µl *w*Bm MetC were incubated in a standard reaction mixture of 80 µl with 50 mM L-alanine and 20 µM pyridoxal phosphate (PLP) in 50 mM KH_2PO_4, 100 mM KCl, pH 8. As a positive control alanine racemase of *B. stearothermophilus* was used. To determine a time course of the enzyme activity samples of the reaction mixture were taken every 60 min over a period of 5 h. To analyze the PLP-dependence of the enzyme different concentrations of PLP (20 – 100 µM) were added and the reaction mixtures were incubated for 4 h. To test the effect of the inhibitor L-aminoethoxyvinylglycine (AVG) 0.4 µg/µl *w*Bm MetC was pre-incubated with increasing concentrations of AVG (0.3 – 0.9 mM). After 30 min substrate was added. The reaction was stopped after 2 h incubation when the enzyme reaction was in the range of initial velocity.

In the second step of the assay, D-alanine was converted to pyruvate by the addition of 2 U/ml D-amino acid oxidase from porcine kidney (DAAO) for 20 min at room temperature. The amount of pyruvate was determined by a colorimetric assay using 2,4-dinitrophenylhydrazin and a pyruvate standard curve as described previously (Milner *et al.* 1976). For this, the reaction mixture and the pyruvate standard were transferred into a 96-well plate. A mixture of 20 µl 0.1 % dinitrophenylhydrazine in 1 N HCL and 100 µl 2 N HCl was added and incubated for 5 min. The color production was detected at 395 nm. The relative enzyme activity was calculated as the maximal amount of pyruvate produced by 2 U/m DAAO incubated with 50 mM D-alanine.

2.2.4 Microscopy

2.2.4.1 Immunofluorescence microscopy

For immunofluorescence microscopy 1.5 x 10^4 *w*ALb B infected and uninfected C6/36 cells were seeded in 8-well culture slides. The cells were incubated at 26 °C for 9 days and supplemented with or without 1 µg/ml ADEP derivatives every 3 days or 512 µg/ml fosfomycin daily. Culture slides were fixed on days 3, 6 and 9 by incubation in 4 % cold paraformaldehyde for 15 min at room temperature followed by 3 wash steps with cold PBS for 5 min. All following steps were performed at room temperature. The fixed cells were permeabilized with 0.25 % Triton X-100 for 15 min followed by a wash step with PBST for 5 min. Blocking was done with 10 % BSA for 30 min and BSA was removed by washing with PBST for 5 min. As primary antibody a rabbit *Wolbachia* FtsZ anti-serum (1:2000 in PBST) (Landmann *et al.* 2012) or a rabbit *w*PAL anti-serum (1:1000 in PBST) (Turner *et al.* 2009) were used. Cells were incubated with the primary antibody overnight at 4 °C in a humid chamber, washed four times for 10 min with PBST and subsequently incubated with diluted goat anti-rabbit Alexa fluor 488 secondary antibody (1:200 in PBST) for 1 h in the dark in a humid chamber. After three wash steps with PBST for 10 min the slides were incubated with 0.25 µg/ml DAPI for 5 min in the dark, embedded in Vectashield mounting medium, and examined with a fluorescence microscope. Pictures were taken using a digital camera (using the same settings for all samples) and sharpness, brightness and contrast of pictures were edited with Adobe Photoshop.

2.2.4.2 Transmission electron microscopy

Wolbachia infected C6/36 cells were grown in 75 cm^2 culture flasks at 26 °C for 9 days and treated daily with 128 µg/ml fosfomycin or every 3 days with 1 µg/ml ADEP 5 while untreated cells served as a control. The C6/36 cells were detached by scraping and harvested at 2,500 g for 8 min at 4 °C. Cell pellets were cautiously suspended in 3 % glutaraldehyde in 0.2 M cacodylate buffer pH 7.35 and incubated at 4 °C for 2 h with constant rotation. To remove the liquid cells were pelleted by centrifugation for 5 min at 6,000 rpm at 4 °C. For the following three wash steps the pellets were suspended in 0.2 M cacodylate buffer, incubated at 4 °C for 10 min and centrifuged again. All following steps were performed in a fume hood and liquids were removed by centrifugation. Contrasting

was performed with 2 % osmium tetroxide in 0.2 M cacodylate buffer (w/v) for 45 min at 4°C with constant rotation. After three wash steps for 10 min at 4 °C in 0.2 M cacodylate buffer the cells were dehydrated in an ethanol series of 30 % ethanol for 7 min, 50 % ethanol for 7 min, 70 % ethanol for 10 min, 90 % ethanol for 10 min, two times 100 % ethanol for 10 min. Residual ethanol in the cell pellet was removed by incubation in propylene oxide three times for 5 min followed by incubation in a 1:1 mixture of propylene oxide and Epon (prepared using the EMbed 812 Kit) for 4 h at room temperature. For embedding cells were suspended in Epon and incubated for 90 min at room temperature under atmospheric air and subsequently polymerized at 60 °C for 48 h. Ultra-thin sections of 60 nm were cut using an ultra-microtome and stained with 3 % uranylacetate (w/v) for 30 min followed by 0.3 % lead citrate (w/v) for 30 min accompanied by extensive washings in demineralized water. The sections were examined with a transmission electron microscope.

2.2.4.3 Immunogold staining for transmission electron microscopy

*w*Alb *B* infected C6/36 cells were grown in plug-sealed 75 cm^2 culture flasks at 26 °C for 9 days with or without 512 µg/ml fosfomycin daily. The insect cells were detached by scraping and harvested at 2,500 g for 8 min at 4 °C. For fixation cell pellets were suspended in 4 % paraformaldehyde and 0.1 % glutaraldehyde in 0.1 M cacodylate buffer, incubated at 4 °C for 2 h and washed three times in 0.1 M cacodylate buffer. The cells were dehydrated in an ethanol series of 30 % ethanol for 7 min, 50 % ethanol for 7 min, 70 % ethanol for 10 min, 90 % ethanol for 10 min and two times 100 % ethanol for 10 min. Subsequently the cells were incubated in a 1:1 mixture of LRWhite and 100 % ethanol overnight at 4 °C. On the next day two incubation steps in LRWhite for 3 h at 4 °C followed. Cells were suspended in fresh LRWhite and the resin was polymerized in gelatin capsules for two weeks at 37 °C. Ultra-thin sections of 80 nm were cut using an ultra-microtome. For immunogold labeling the sections were quenched with 0.05 M glycine in TBS (pH 8.2), blocked in a mixture of 0.5 % goat serum, 5 % BSA and 0.1 % sodium azide (w/v) in TBS (pH 8.2) and washed with wash buffer two times for 3 min. As primary antibody either a rabbit *Wolbachia* FtsZ anti-serum diluted 1:50 in wash buffer or a rabbit *w*PAL anti-serum diluted 1:5 in wash buffer was used. After incubation for 24 h in a humid chamber at 4 °C the sections were washed in wash buffer seven times for 3 min followed

by an incubation step for 24 h at 4 °C in a humid chamber with a goat anti-rabbit gold conjugate (10 nm) second antibody diluted 1:40 in wash buffer. After washing seven times for 3 min in wash buffer sections were post-fixed in 2 % glutaraldehyde and washed once with demineralized water. Previous to the examination with a transmission electron microscope sections were stained with 3 % uranylacetate (w/v) for 30 min followed by 0.3 % lead citrate (w/v) for 30 min followed by extensive washings in demineralized water.

3 Results

3.1 Characterization of lipid II biosynthesis in *Wolbachia* and its impact on cell division

3.1.1 Transcription analysis of genes involved in the lipid II metabolism

The *Wolbachia* genome harbors many genes involved in lipid II metabolism as discovered by genome annotation (Foster *et al.* 2005). The enzymatic activity of MraY and MurG that catalyze the last two, membrane bound steps of lipid II synthesis from UDP-MurNAc pentapeptide was previously shown in *Wolbachia* (Henrichfreise *et al.* 2009). To confirm the transcription of all other genes involved in lipid II metabolism in arthropod *Wolbachia*, their expression was analyzed at the RNA level. RNA was isolated from an *A. albopictus* C6/36 insect cell line infected with *w*Alb B, as well as from an uninfected C6/36 cell line as negative control. In the first step the RNA was reverse transcribed using random decamer primers. In the second step fragments of around 200 – 300 bp of the genes of interest were amplified using gene specific primers. The PCR products were separated on 2 % agarose gels and visualized with a fluorescent DNA binding dye. To exclude contamination by genomic DNA a second negative control without reverse transcriptase was added. As PCR positive control genomic DNA of *w*Alb B infected C6/36 cells was used. All results were confirmed by sequencing.

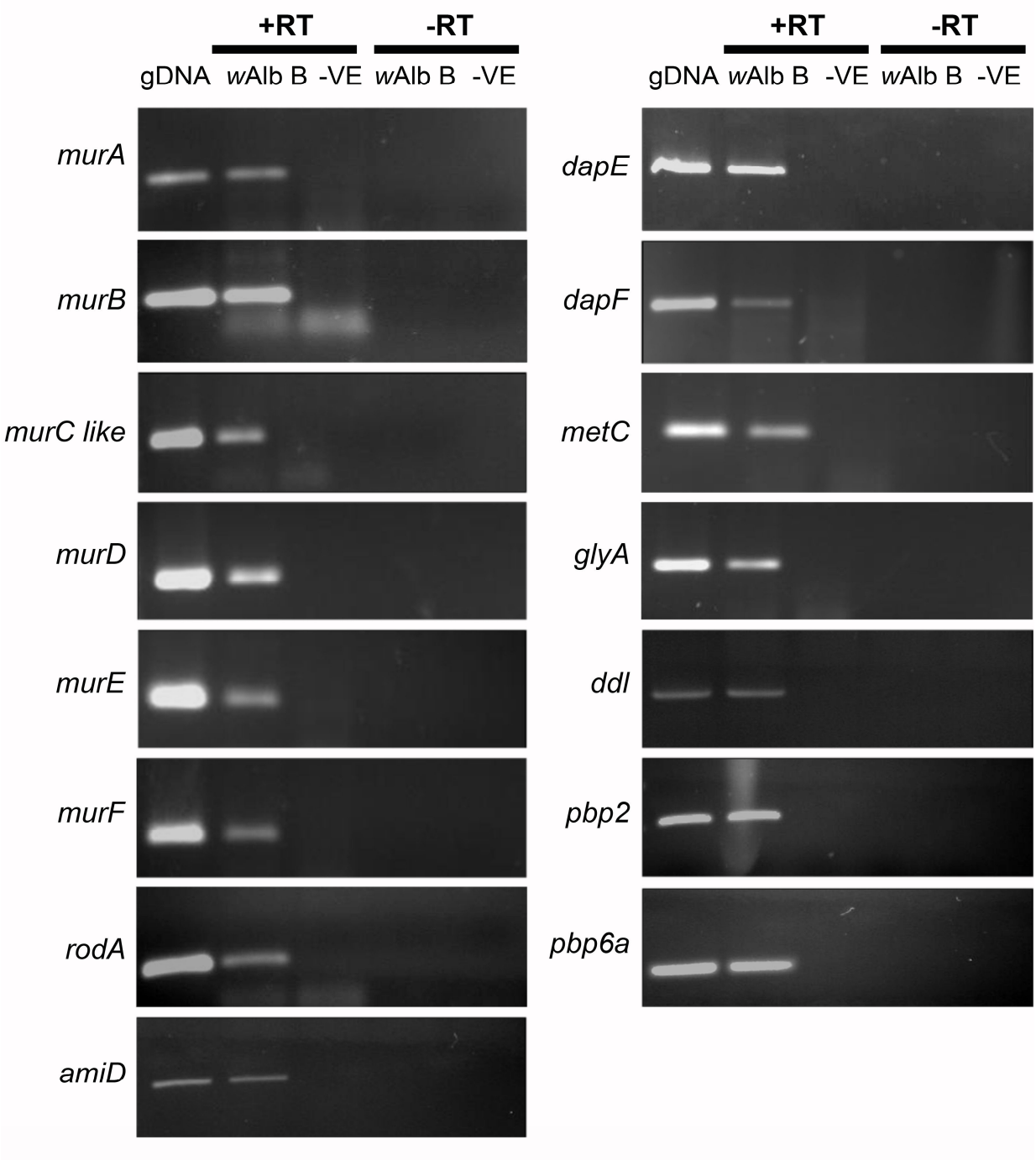


Figure 3.1 Expression of genes involved in the lipid II metabolism in *Wolbachia* strain *w*Alb B (Vollmer, *J. et al.* 2013). PCR products were examined on 2 % agarose gels. RNA was isolated from a *Wolbachia* infected (*w*Alb B) and an uninfected (-VE) C6/36 insect cell line. Two-step RT-PCR was carried out using random decamer primers and gene specific primers, respectively. DNA contamination was monitored by adding a negative control without reverse transcriptase. All results were confirmed by sequencing. RT-PCRs for the genes *murA* – *murF*, *rodA*, *metC* and *glyA* were performed by Karen Jülicher as part of her bachelor thesis (Jülicher 2012). gDNA: genomic DNA, RT: Reverse transcriptase.

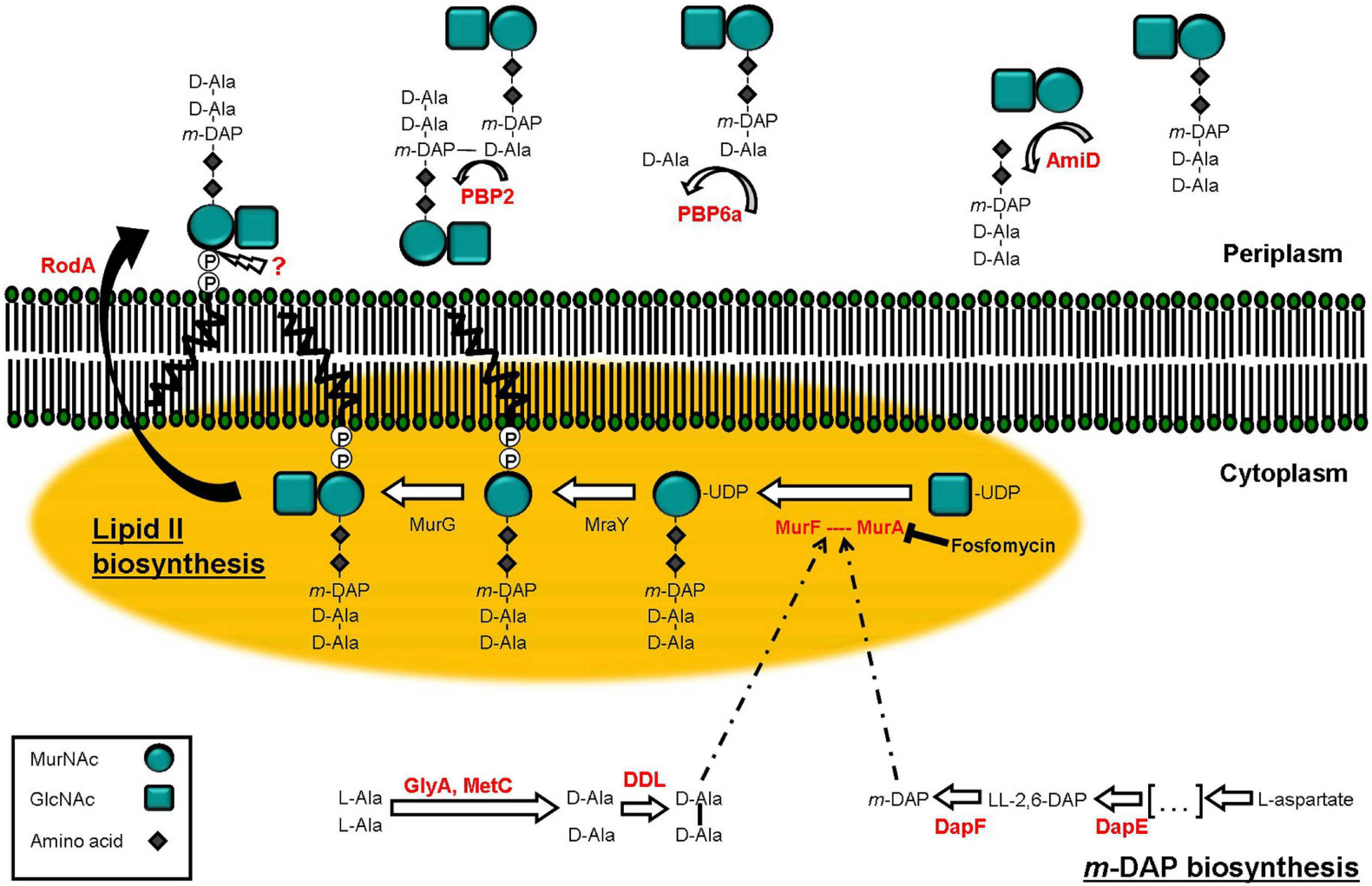


Figure 3.2: Model of peptidoglycan biosynthesis in *Wolbachia* based on those genes whose transcription was confirmed by RT-PCR (depicted in red) (Vollmer, J. *et al.* 2013). The functionality of the enzymes MraY and MurG was shown previously (Henrichfreise *et al.* 2009). The question mark indicates catalytic steps, which remain to be elucidated. Lipid II biosynthesis takes place in the cytoplasm. *Meso*-DAP and D-alanine dipeptide are supposed to built the terminus of the peptide side chain. Subsequent to translocation across the membrane lipid II might be further processed via peptide cross-links.

By this method, the transcription of the genes *murA* to *murF* could be confirmed in the *w*Alb B infected cells (Figure 3.1). Commonly, these enzymes catalyze the formation of UDP-MurNAc-pentapeptide from UDP-GlcNAc (Figure 3.2). UDP-MurNAc-pentapeptide is then converted to lipid II by the enzymes MurG and MraY and it could be shown previously that the these two enzymes are functional *in vitro* and *ex vivo* (Henrichfreise *et al.* 2009). Furthermore, the transcription of the genes *dapE* and *dapF* could be shown by RT-PCR (Figure 3.1). The products of these genes catalyze the last two steps of the synthesis of *meso*-DAP, which is found at position three of the peptide side chain of Gram-negative bacteria. In most bacteria a D-alanine dipeptide is linked to the nascent tripeptide in the final step of UDP-MurNAc-pentapeptide synthesis. *Wolbachia* possess the gene for the D-alanine ligase DDL, but no known D-amino acid racemases were discovered (Foster *et al.* 2005). Recently, the enzymes MetC and GlyA, normally involved in methionine biosynthesis, were demonstrated to have D-alanine racemase activity in *E. coli* (Kang *et al.* 2011). The genes for MetC and GlyA could be found in every *Wolbachia* genome sequenced so far (Table 3.1) while all other genes of the methionine biosynthesis pathway are missing. By RT-PCR the genes *metC, glyA* and *ddl* were investigated and as a result the transcription of all three genes could be confirmed (Figure 3.1).

After synthesis in the cytoplasm, lipid II is flipped into the periplasm where it is incorporated into the nascent peptidoglycan network via glycosyltransferases and transpeptidases. Every *Wolbachia* strain possesses a gene encoding a potential lipid II flippase of the SEDS family and two or three PBPs (Table 3.1) but their genomes lack glycosyltransferases and almost all genes of the peptidoglycan degradation and recycling pathway. By RT-PCR the transcription of genes encoding the potential flippase RodA, PBP2, PBP6a and an amidase of the degradation pathway, AmiD, could be confirmed in wAlb B (Figure 3.1).

Table 3.1: Potential amino acid racemases and lipid II translocating and processing enzymes in *Wolbachia*

	Wolbachia **strain**			
	***w*Pa**[1]	***w*Bm**[2]	***w*Mel**[3]	***w*Ri**[4]
GlyA [5]	YP_001975594 (52%, 4,0E-150)[8]	YP_197839 (55%, 1,0E-148)	NP_966759 (54%, 2,0E-153)	YP_002727560 (54%, 1E-152)
MetC[5]	YP_001974958 (35%, 5E-83)	YP_198323 (36%, 1E-86)	NP_966663 (35%, 8E-84)	YP_002727398 (35%, 9E-83)
FtsW[6]	-[9]	YP_197849 (31%, 2E-38)	-	-
RodA[6]	YP_001975766 (38%, 1E-60)	-	NP_966828 (36%, 3E-60)	YP_002727752 (38%, 5E-62)
PBP2[7]	YP_001975343.1 (29%, 2E-79)	YP_197909.1 (28%, 1E-78)	NP_966477.1 (30%, 7E-84)	YP_002727216.1 (30%, 1E-84)
PBP3[7]	-	-	NP_966983.1 (26%, 7E-54)	YP_002727717.1 (26%, 5E-54)
PBP6a[7]	YP_001976086.1 (34%, 2E-64)	YP_198121.1 (35%, 2E-65)	NP_965921.1 (35%, 3E-66)	YP_002726790.1 (34%, 2E-65)

[1] *w*Pa: *Wolbachia pipientis* strain endosymbiont of *C. quinquefaciatus Pel* (GenBank accession AM999887.1). BLAST analysis was performed using the genome sequence of the closest related strain *w*Pa (Mavingui *et al.* 2012) since the whole genome sequence of *w*Alb B was not available until the time of investigation.
[2] *w*Bm: *Wolbachia* endosymbiont of *B. malayi* (GenBank accession AE017321.1).
[3] *w*Mel: *Wolbachia pipientis* endosymbiont of *D. melanogaster* (GenBank accession AE017196.1).
[4] *w*Ri: *Wolbachia pipientis* endosymbiont of *D. simulans* strain Riverside (GenBank accession CP001391.1).
[5] GlyA and MetC have an alternative racemase activity in *E. coli* (Kang *et al.* 2011).
[6] Potential lipid II translocating proteins.
[7] Potential lipid II processing proteins.
[8] NCBI BLASTP sequence identities and expected (E) values of alignments between the *Wolbachia* protein sequences and the respective protein in *E. coli* str. K-12 substr. W3110 (GenBank accession AP009048.1).
[9] Dashes depict genes that are absent from the genome.

3.1.2 Investigation of the racemase activity of recombinant *Wolbachia* MetC

Characteristic for the cell wall of all prokaryotes investigated is the occurrence of D-amino acids in the peptide moieties of peptidoglycan and its precursors. Although

Wolbachia lack the genes for all known racemases they might be able to convert L-amino acid to D-amino acid via the enzymes MetC and GlyA which were shown to exhibit an alternative L-alanine racemase activity in *E. coli* (Kang *et al.* 2011) and that are transcribed in *Wolbachia* (section 3.1.1). To investigate the L-alanine racemase activity of MetC from *Wolbachia* strain *w*Bm, recombinant *w*Bm MetC was overexpressed in *E. coli* strain BL21 and purified by Ni-NTA affinity chromatography. Cloning of the *w*Bm *metC* gene and optimization of basic assay conditions were performed as part of a bachelor thesis supervised during this PhD project (Jülicher 2012). The concentration of eluted recombinant protein was determined by Bradford assay and 0.2 µg/µl protein was analyzed by SDS-PAGE.

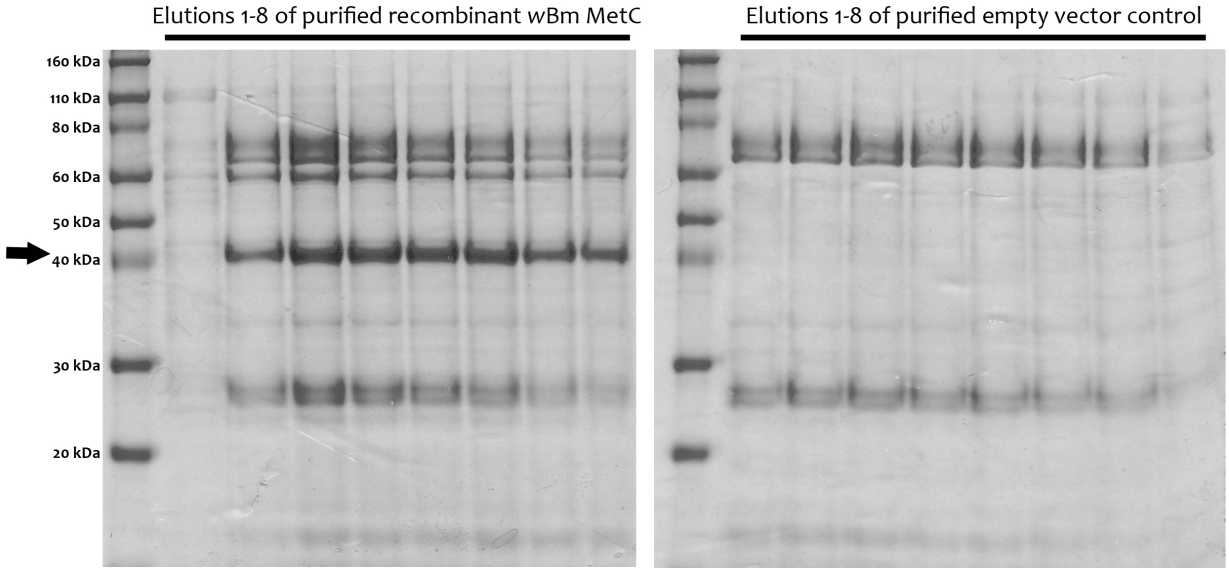


Figure 3.3: Separation of purified recombinant protein by SDS-PAGE. 0.2 µg/µl protein of purified recombinant *w*Bm MetC and empty vector control, respectively, were applied to SDS-PAGE, subsequently stained in Coomassie staining solution and destained in 30 % methanol. The arrow depicts the protein band of *w*Bm MetC.

In contrast to the empty vector control, elutions of purified recombinant *w*Bm MetC exhibited a strong protein band of between 40 and 50 kDa size that correlated to the size of His-tagged *w*Bm MetC of 47 kDa (Figure 3.3). Furthermore a second band of around 60 kDa was exclusively visible in the elutions containing purified *w*Bm MetC but not in the empty vector control. All samples of both purifications also contained several secondary bands of which the most prominent were found between 20 - 30 kDa and 60 – 80 kDa, respectively.

The racemase activity of purified protein was analyzed in a coupled enzymatic assay. For this, 0.4 µg/µl protein was incubated in a reaction mixture containing the cofactor pyridoxal phosphate (PLP) and L-alanine. In a second step D-amino acid oxidase from porcine kidney (DAAO) was added which specifically converts D-alanine to pyruvate. The amount of pyruvate was finally determined using a colorimetric assay including a pyruvate standard curve. The relative enzyme activity was calculated as the maximal amount of pyruvate produced by 2 U/m DAAO incubated with 50 mM D-alanine. To exclude contamination by native racemases from the *E. coli* expression strain, racemase activity of the empty vector control elution fractions was also determined. As a positive control alanine racemase of *B. stearothermophilus* was used.

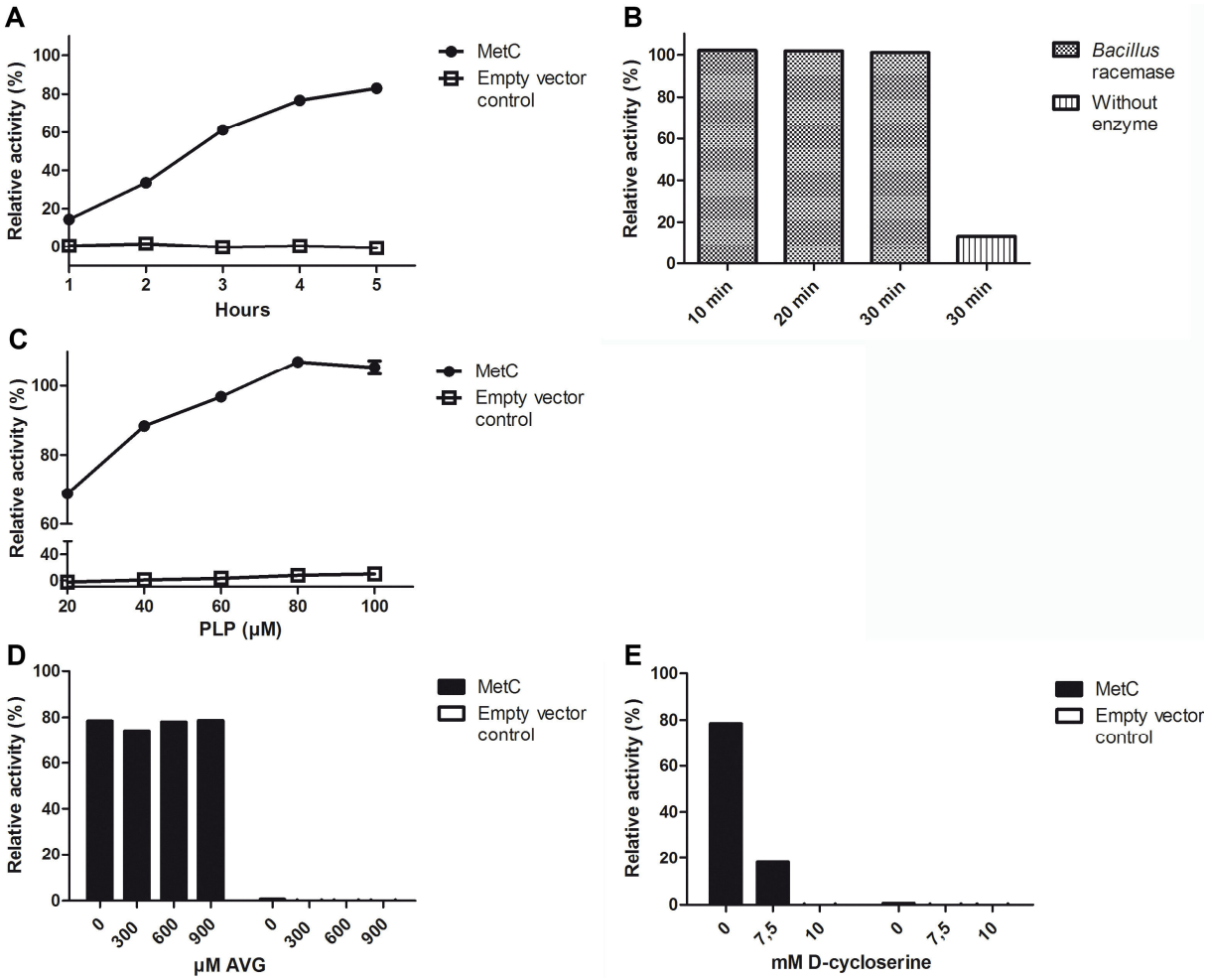


Figure 3.4: D-alanine racemase activity of *Wolbachia* MetC. The D-alanine racemase activity was investigated in a coupled enzymatic assay using recombinant MetC of *w*Bm, alanine racemase of *B. stearothermophilus* and an empty vector control, respectively. In the first step 0.4 µg/µl protein was incubated with the cofactor PLP and 50 mM L-alanine. Same volumes of the empty vector control were used in this assay as negative control. In the second step DAAO was added which specifically converted produced D-alanine to pyruvate. The pyruvate concentration of the samples was detected using a colorimetric assay. The relative enzyme activity was normalized to the maximal amount of pyruvate produced by DAAO incubated with D-alanine. The data shown are representative of three experiments. **(A)**

Enzyme activity at different time points in an assay containing 20 µM PLP. **(B)** Enzyme activity of *B. stearothermophilus* alanine racemase at different time points in an assay containing 20 µM PLP. **(C)** Enzyme activity after 4 h incubation in an assay containing different concentrations of PLP. **(D)** Effect of the inhibitor AVG on the enzymatic activity of MetC. The protein was pre-incubated for 30 min with 20 µM PLP and different concentrations of AVG. The reaction was started by the addition of the substrate and proceeded for 2 h. **(E)** Impact of the inhibitor D-cycloserine on the enzymatic activity of MetC. The protein was pre-incubated for 30 min with 20 µM PLP and different concentrations of D-cycloserine. The reaction was started by the addition of the substrate and proceeded for 2 h.

In this assay L-alanine was converted to D-alanine by *w*Bm MetC thus demonstrating the L-alanine racemase activity of the enzyme, while no activity was observed in the empty vector control (Figure 3.4 A). The enzymatic reaction was time-dependent and proceeded over hours (Figure 3.4 A). After 5 hours of incubation a relative activity of around 80 % was achieved. In comparison, using the same concentration of an alanine racemase from *B. stearothermophilus* 100 % of relative enzyme activity was reached after 10 minutes (Figure 3.4 B). The enzymatic activity increased due to the supplementation of the cofactor PLP in a concentration dependent manner and the addition of 80 µM PLP resulted in 100 % relative activity after 4 hours of incubation (Figure 3.4 C).

The original reaction catalyzed by MetC in methionine biosynthesis is the conversion of L-cystathionine to L-homocysteine. In *E. coli* this catalytic step is inhibited by the compound L-aminoethoxyvinylglycine (AVG) due to the binding of the active site of the enzyme (Clausen *et al.* 1997). To test whether AVG is also able to inhibit the racemase activity of *w*Bm MetC, the enzyme was incubated with increasing concentrations of inhibitor. The L-cystathionine converting enzymatic activity of *E. coli* MetC was shown to decrease dramatically after the addition of 300 µM AVG (Clausen *et al.* 1997). In contrast, *w*Bm MetC did not show any loss of relative enzyme activity even at 3-fold higher concentrations of AVG (Figure 3.4 D). An inhibitor of the known bacterial alanine racemases is the antibiotic D-cycloserine that acts as a substrate analog of D-alanine and covalently binds to the cofactor PLP. To determine the inhibitory effect of D-cycloserine on *w*Bm MetC the enzyme was incubated with different concentrations of inhibitor. The relative enzyme activity of *w*Bm MetC incubated with 7.5 mM D-cycloserine was strongly reduced and at a concentration of 10 mM D-cycloserine no activity was left (Figure 3.4 E). However, D-cycloserine complexes not only enzyme-bound PLP but free PLP as well (Manohar *et al.* 1984). Since the racemase activity of *w*Bm MetC is dependent

on the addition of PLP it could not be distinguished in this assay whether D-cycloserine is able to bind *w*Bm MetC or whether the activity was affected by the depletion of free PLP.

Whether the GlyA enzyme also exhibits racemase activity in *Wolbachia* could not be investigated since recombinant *w*Bm GlyA has not been achieved.

3.1.3 Phenotypic effect of lipid II depletion by fosfomycin treatment

It has been shown that *Wolbachia* are sensitive to fosfomycin (Henrichfreise *et al.* 2009), a specific inhibitor of MurA (Figure 3.2). To elucidate the effect of inhibition on *Wolbachia,* the phenotype of the treated bacteria was examined by microscopy. For this, *Wolbachia* infected C6/36 cells were seeded in culture slides, treated with fosfomycin for 9 days and examined by fluorescence microscopy. *Wolbachia* were visualized by staining their DNA using DAPI as well as by a specific immunofluorescence staining using anti-serum against *Wolbachia* FtsZ (Landmann *et al.* 2012) or *w*PAL (Turner *et al.* 2009) and an alexa 488 conjugated secondary antibody.

In the fosfomycin treated insect cells the amount of *Wolbachia* was strongly reduced compared to the untreated control (Figure 3.5). The treated insect cells stained with DAPI exhibited large, uniformly-shaped DNA containing objects in their cytoplasm (Figure 3.5B). Immunofluorescence staining using *Wolbachia* specific antibodies demonstrated that those large objects are *Wolbachia* bacteria showing a highly aberrant phenotype (Figure 3.5B, D). Fosfomycin treated *Wolbachia* furthermore showed an altered localization pattern of the protein *w*PAL (Figure 3.5C, D). *w*PAL is the homolog of the lipoprotein Pal that non-covalently binds the peptidoglycan of Gram-negative bacteria (see section 1.1.1). In untreated *Wolbachia*, *w*PAL was distributed over the whole cell surface whereas fosfomycin treated bacteria seemed to have less *w*PAL with a dot-like staining pattern indicating an accumulation of *w*PAL to certain spots (Figure 3.6D).

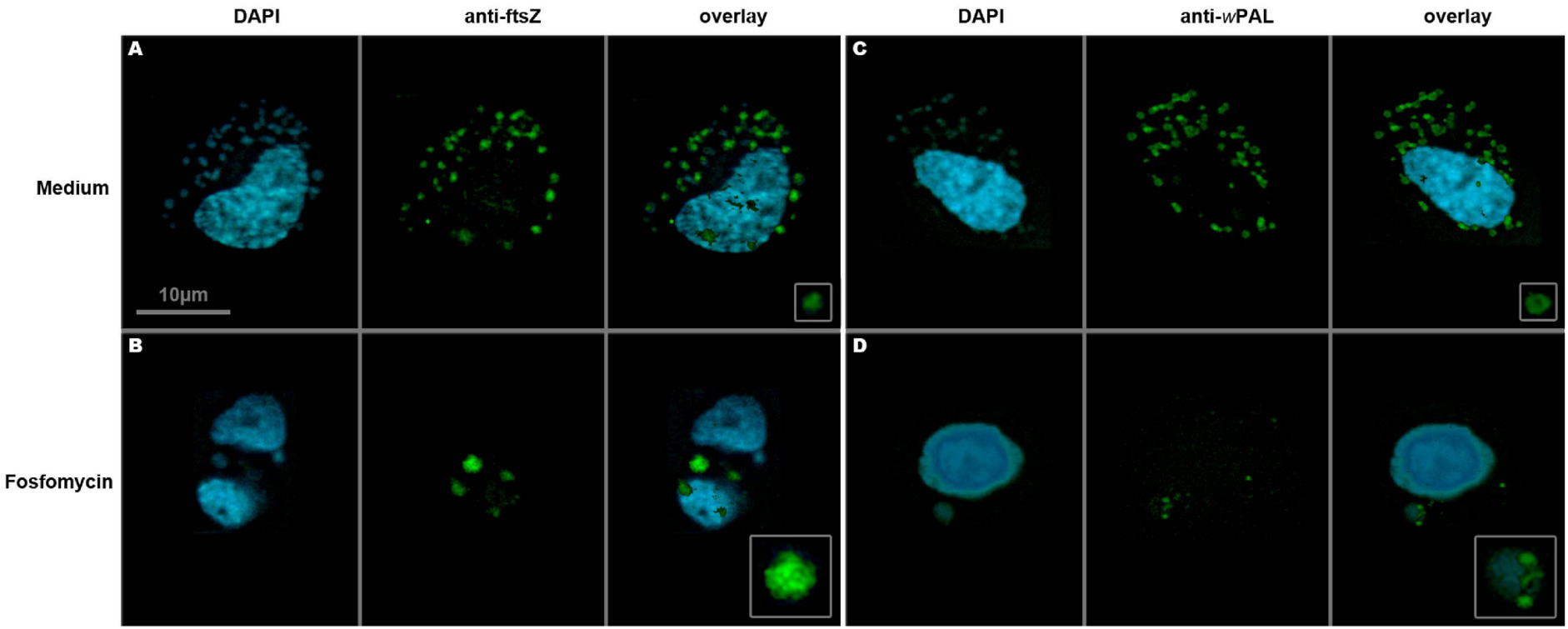


Figure 3.5: Enlargement of *Wolbachia* bacteria after inhibition of the lipid II synthesis (Vollmer, J. *et al.* 2013). C6/36 insect cells infected with *w*Alb B were incubated with fosfomycin (B, D) or without antibiotic (A, C). Fixed cells were examined by immunofluorescence microscopy using FtsZ anti-serum (A, B) or *w*PAL anti-serum (C, D) and an alexa 488 conjugated secondary goat anti-rabbit antibody (green). DNA of *Wolbachia* and insect cell nuclei were stained with DAPI (blue). Small boxes: 2.5 x image enlargements of *Wolbachia* bacteria.

It could not be differentiated by fluorescence microscopy whether these single large objects represented an accumulation of several *Wolbachia* or one single, enlarged *Wolbachia* cell. Therefore the ultrastructure of fosfomycin treated and untreated *w*Alb B infected insect cells was examined by transmission electron microscopy. Infected cells were grown with or without the addition of fosfomycin for 9 days, harvested, fixed in glutaraldehyde and osmium teroxide, subsequently dehydrated in an ethanol series and finally embedded in Epon. Ultra-thin sectionswere stained with uranyl actetate and lead citrate.

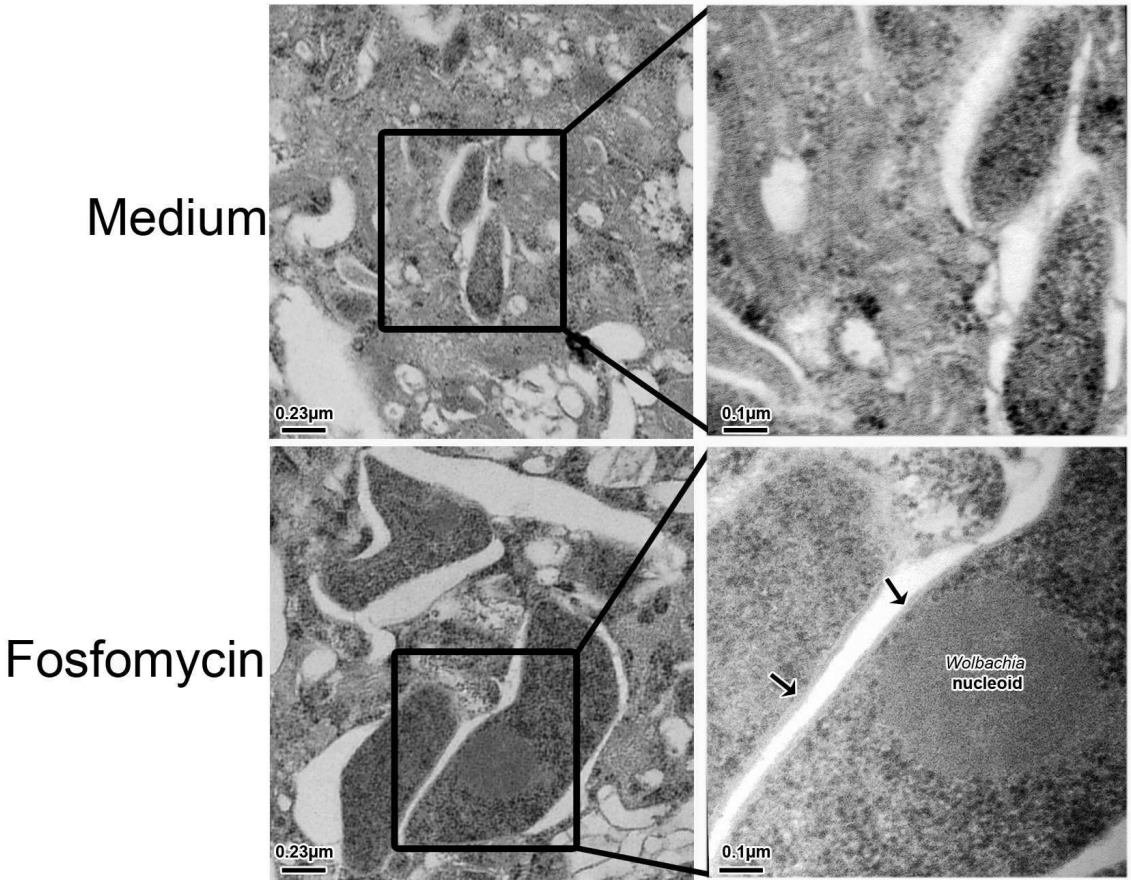


Figure 3.6: Transmission electron microscopy of fosfomycin treated and untreated *Wolbachia* (Vollmer, J. *et al.* 2013). C6/36 insect cells infected with *w*Alb B were incubated with or without fosfomycin for 9 days, fixed in glutaraldehyde, postfixed in osmium tetroxide, dehydrated in an ethanol series and embedded in Epon. Ultra-thin sections were stained using uranyl acetate and lead citrate. Black boxes mark those areas which were examined in higher magnifications. Arrows depict cell membranes of enlarged *Wolbachia*.

In untreated insect cells, *Wolbachia* exhibited an amorphous, electron dense morphology and were located in vacuoles (Figure 3.6). Ribosomes were visible as small dark dots in *Wolbachia* as well as in insect cells. In some cases the bacterial nucleoids could be observed as smooth, round shaped structures. Compared to the untreated

control in the fosfomycin treated insect cells no accumulation but a strongly enlarged morphology of amorphous *Wolbachia* cells inside vacuoles could be observed, some also with large nucleoids. Although fosfomycin is an inhibitor of the first step of cell wall biosynthesis the *Wolbachia* membranes were not seriously affected by the treatment. No cell lysis or membrane disruptions could be observed.

3.1.4 Impact of acyldepsipeptide treatment on *Wolbachia*

Antibiotics of the acyldepsipeptide (ADEP) class are highly active against Gram-positive bacteria whereas no natural sensitivity of Gram-negative bacteria has been observed since the size of the molecule is too large to enter the outer membrane (Brötz-Oesterhelt *et al.* 2005) (Figure 6.1). However, *Wolbachia* were strongly depleted subsequent to treatment with ADEPs as demonstrated by quantitative real-time PCR (qPCR) ((Schiefer *et al.* 2012, submitted), personal communiation) perhaps due to the fact that they do not possess the classical Gram-negative outer membrane (Foster *et al.* 2005). ADEPs induce the dysregulation of the bacterial caseinolytic Clp protease, an enzyme responsible for the degradation of misfolded or damaged proteins (Lee *et al.* 2010). Binding of ADEP molecules to the protease results in a permanent and uncontrolled proteolytic activity (Brötz-Oesterhelt *et al.* 2005). It was demonstrated in strains of *Bacillus* and *Streptococcus* that especially the major cell division protein FtsZ is a target for degradation. As a consequence, proper cell division was impaired, leading to filamentation and swelling of the bacterial cells (Sass *et al.* 2011).

Interestingly a similar phenotype was observed in *Wolbachia* after blocking the synthesis of lipid II (see section 3.1.3). Therefore the phenotypic effect of ADEP treatment on *Wolbachia* was examined by immunofluorescence microscopy to investigate whether treatment leads to depletion of FtsZ and impairment of cell division as seen in Gram-positive bacteria. For this, ADEP treated *w*Alb B infected C6/36 insect cells were seeded in culture slides and incubated with or without different ADEP derivatives for 9 days. Fixed cells were stained using the DNA stain DAPI as well as using *Wolbachia* FtsZ anti-serum (Landmann *et al.* 2012) in combination with an alexa 488 conjugated secondary antibody.

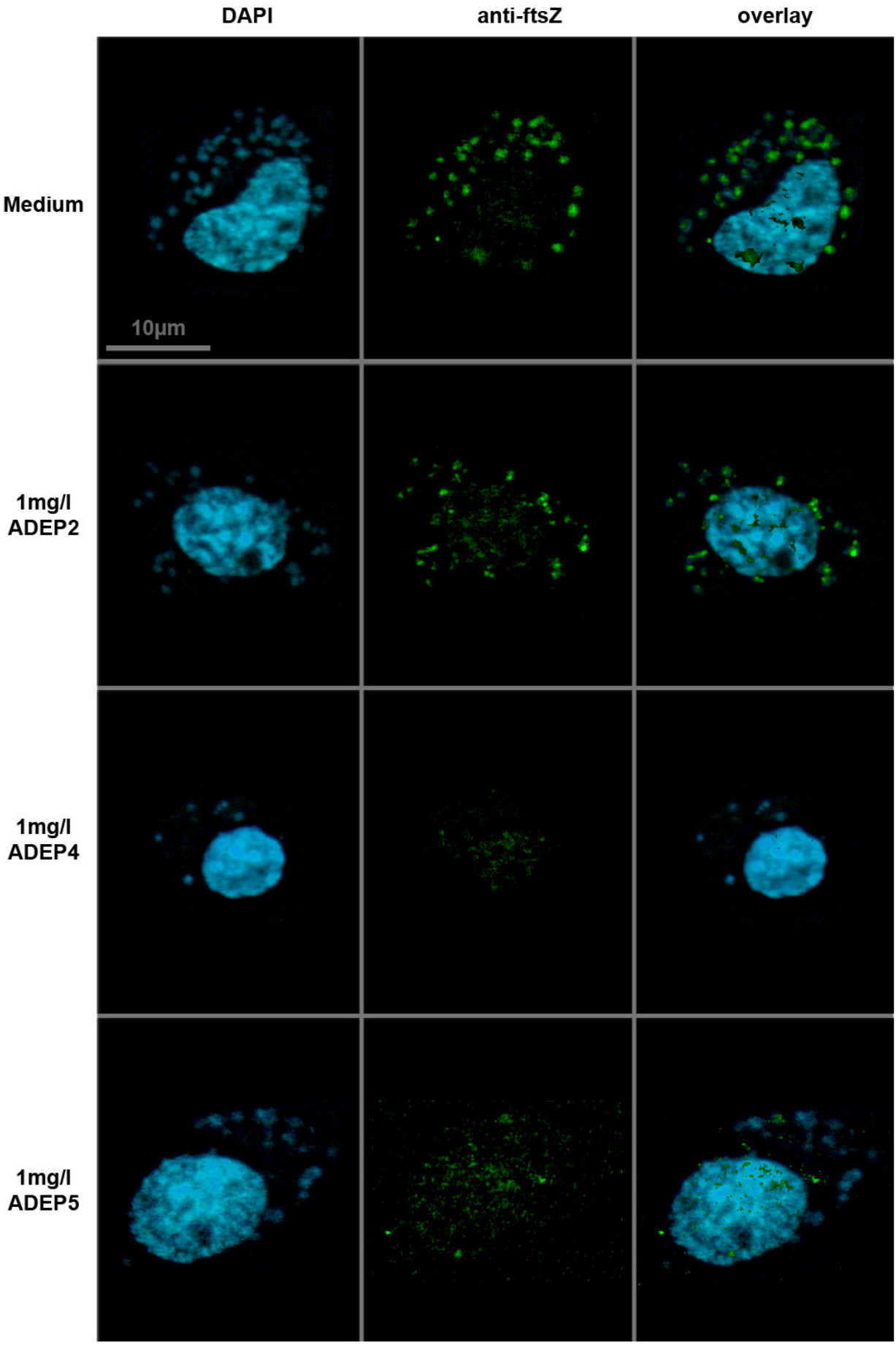
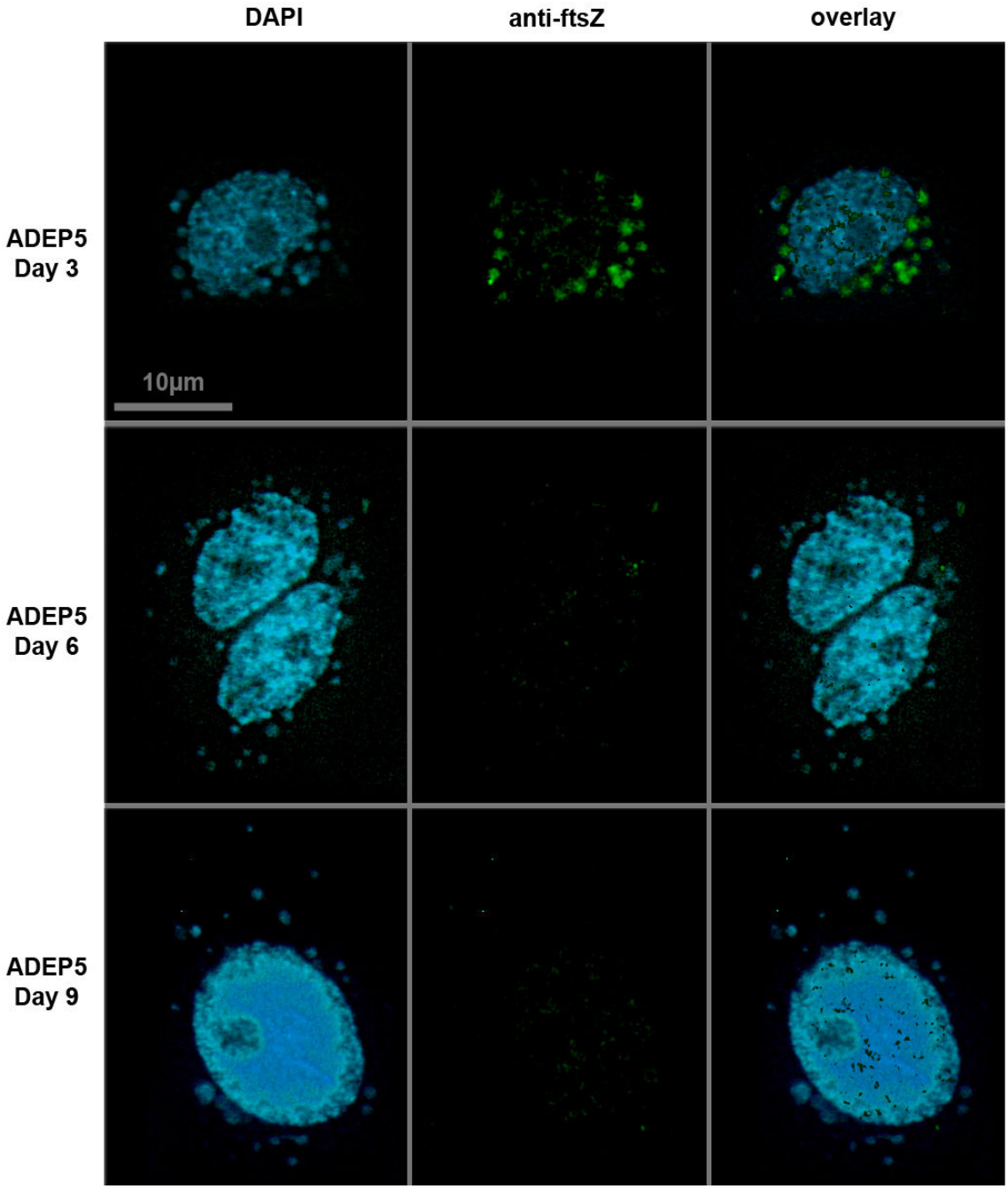

DAPI
anti-ftsZ
overlay
Medium
10µm
1mg/l ADEP2
1mg/l ADEP4
1mg/l ADEP5

Figure 3.7: Impact of treatment with ADEP derivatives on the phenotype of *Wolbachia* (Schiefer *et al.* 2012, submitted). *w*Alb B infected insect cells were incubated with ADEP 2, ADEP 4, ADEP 5 (for molecular structures s. Figure 6.1) or without antibiotic for 9 days. Fixed cells were visualized by immunofluorescent staining using FtsZ anti-serum and an alexa 488 conjugated secondary antibody (green). DNA of *Wolbachia* and insect cell nuclei were stained with DAPI (blue).

After 9 days insect cells incubated with ADEP 2 or without antibiotic were highly infected with *Wolbachia* and exhibited an extensive anti-FtsZ staining that colocalized with the DNA staining of *Wolbachia* cells (Figure 3.7). In contrast, insect cells treated with ADEP 4 and ADEP 5 were less infected with *Wolbachia* and *Wolbachia* cells visualized by DNA staining lacked any anti-FtsZ staining except few weak spots that did not colocalize with the DAPI staining. However, in FtsZ depleted cells no cell enlargement or other morphological aberrations could be observed compared to the control.

To further investigate the depletion of FtsZ protein in *Wolbachia* cells subsequent to ADEP treatment a time course analysis was performed. *w*Alb B infected insect cells were incubated with ADEP 5 or without antibiotic for 9 days. Cells were fixed on day 3, day 6 and day 9 and were stained as described above. Subsequent to the treatment with ADEP 5 for 3 days a strong FtsZ staining was observed that colocalized with the DNA staining of *Wolbachia* cells. After 6 and 9 days of treatment FtsZ was depleted (Figure 3.8). At neither time point was any aberrant morphology seen.

Figure 3.8: Time course analysis of the effect of ADEP 5 treatment on *Wolbachia* (Schiefer *et al.* 2012, submitted). *w*Alb B infected insect cells were incubated with ADEP 5 or without antibiotic for 9 days. Cells were fixed on day 3, day 6 and day 9 and were visualized by immunofluorescent staining using FtsZ anti-serum and an alexa 488 conjugated secondary antibody (green). DNA of *Wolbachia* and insect cell nuclei were stained with DAPI (blue).



It was demonstrated in this PhD project that the depletion of lipid II in *Wolbachia* resulted not only in the enlargement of *Wolbachia* cells but furthermore in an altered localization pattern of *w*PAL protein indicating the participation of *w*PAL in cell division (section 3.1.3). Therefore the effect of ADEP treatment on *w*PAL localization was elucidated. *w*Alb B infected insect cells were treated with ADEP 5 for 9 days and visualized by immunofluorescent staining as described above and staining using *w*PAL anti-serum (Turner *et al.* 2009) and an alexa 488 conjugated secondary antibody.

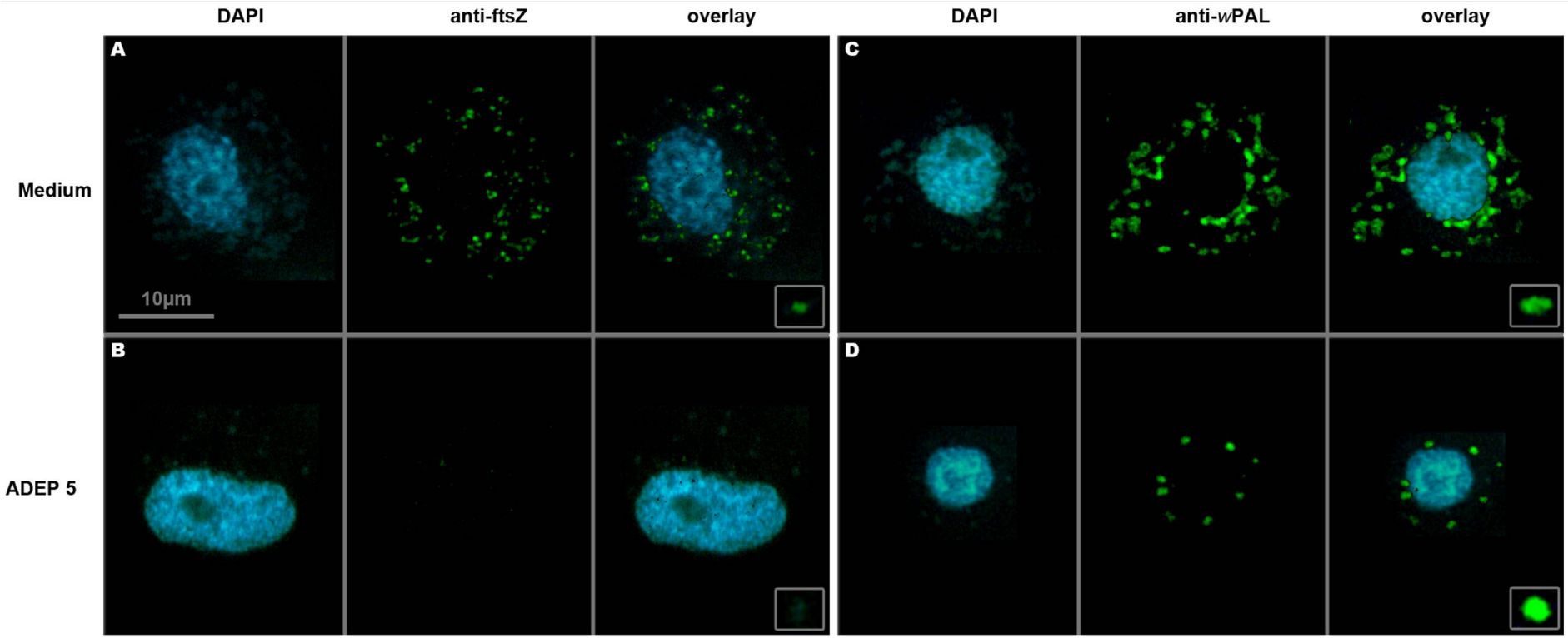


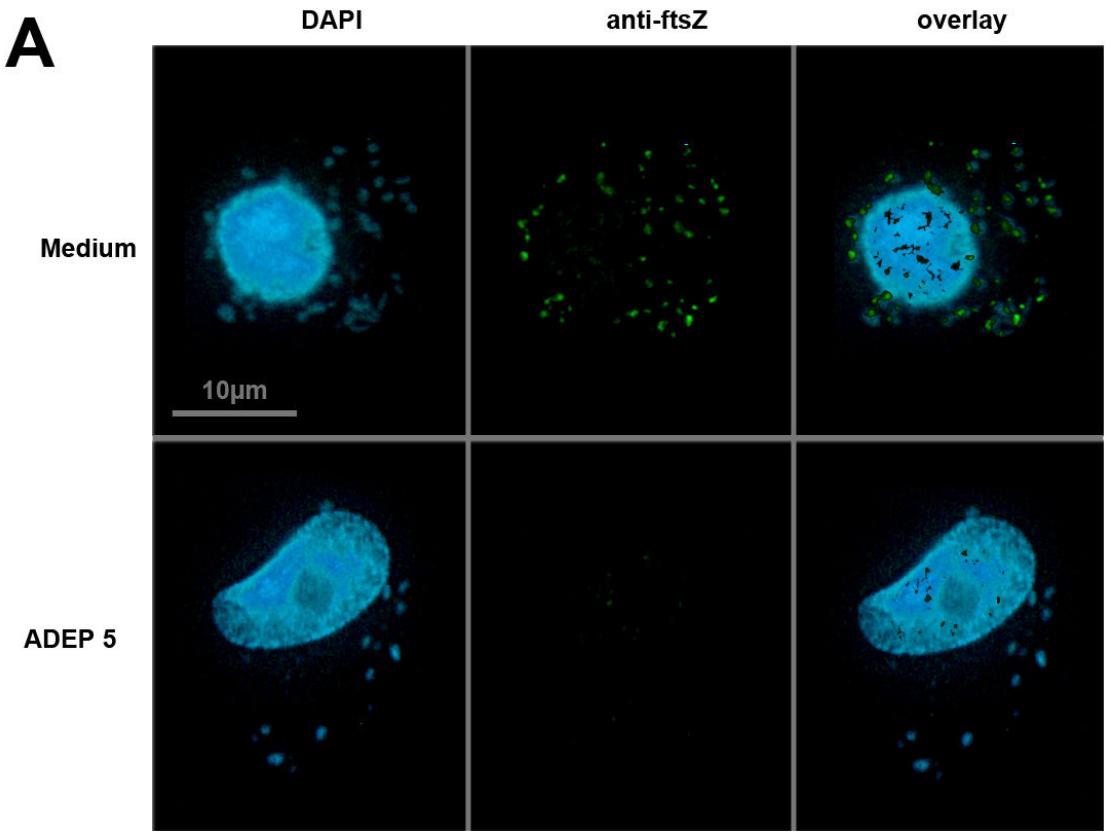
Figure 3.9: FtsZ depletion in *Wolbachia* by ADEP 5. C6/36 insect cells infected with *w*Alb B were incubated with ADEP 5 (B, D) or without antibiotic (A, C). Fixed cells were examined by immunofluorescence microscopy using anti-FtsZ antiserum (A, B) or anti-*w*PAL antiserum (C, D) and an alexa 488 conjugated secondary goat anti-rabbit antibody (green). DNA of *Wolbachia* and insect cell nuclei were stained with DAPI (blue). Small boxes: 2.5 x image enlargements of *Wolbachia* bacteria.

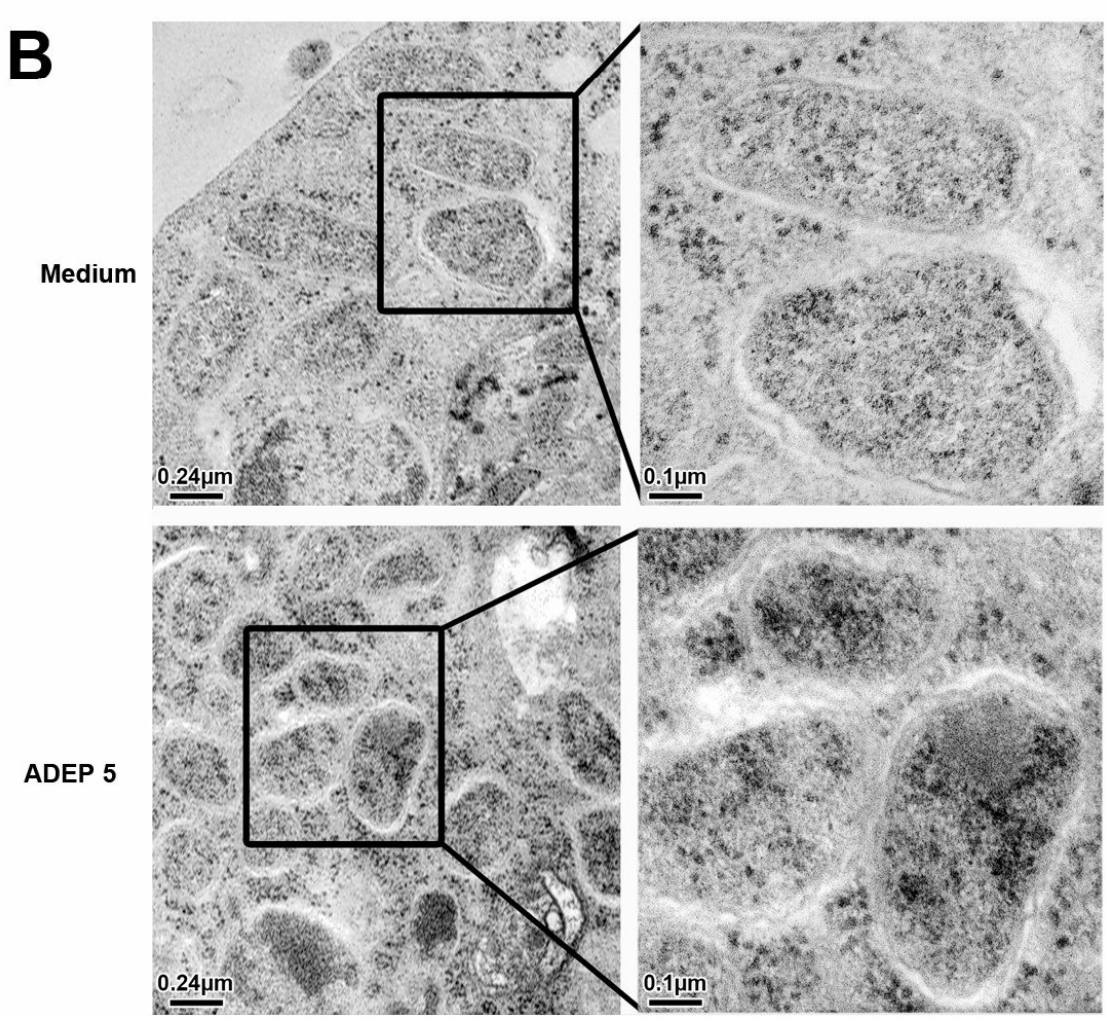
After 9 days of ADEP 5 treatment depletion of FtsZ in *Wolbachia* cells was observed whereas a strong ftsZ singal could be detected in the untreated control. However, the fluorescent staining of *w*PAL did not differ between treated and untreated bacteria and exhibited a rather uniformly, cell surrounding pattern (Figure 3.9).

Although FtsZ is significantly depleted in ADEP 4 and ADEP 5 treated *Wolbachia* no obvious morphological effects like enlargement of cells due to division defects as seen in other bacteria could be observed by immunofluorescence microscopy. To investigate the morphological effects of ADEP treated *Wolbachia* in more detail, *Wolbachia* infected insect cells were examined by transmission electron microscopy. The cells were incubated with and without ADEP 5 for 9 days. To control the depletion of FtsZ subsequent to ADEP 5 treatment a subset of cells was analyzed by immunofluorescence microscopy as described above. For transmission electron microscopy the cells were fixed in glutaraldehyde and osmium tetroxide, dehydrated in an ethanol series and embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate.

Subsequent to the treatment with ADEP 5 fewer *Wolbachia* infected insect cells were found compared to the untreated control. FtsZ was depleted in treated cells but not in the control (Figure 3.10A). However, using transmission electron microscopy in *Wolbachia* treated with ADEP5 no significant morphological differences could be observed. Neither an increase of cell size nor any other aberrant effects were seen (Figure 3.10B).

Figure 3.10: Phenotypic effect of ADEP 5 treatment on *Wolbachia*. C6/36 insect cells infected with *w*Alb B were incubated with or without ADEP 5 for 9 days. **(A)** Fixed cells were analyzed by immunofluorescence using FtsZ anti-serum and an alexa 488 conjugated secondary goat anti-rabbit antibody (green) and DAPI (blue), respecively. **(B)** (Schiefer *et al.* 2012, submitted) For TEM cells were fixed in glutaraldehyde, postfixed in osmium tetroxide, dehydrated in an ethanol series and embedded in Epon. Ultra-thin sections were stained using uranyl acetate and lead citrate. Black boxes mark those areas which were examined in higher magnifications.


A
DAPI
anti-ftsZ
overlay
Medium
10µm
ADEP 5


B
Medium
0.24µm
0.1µm
ADEP 5
0.24µm
0.1µm

3.1.5 Examination of the FtsZ and *w*PAL distribution in dividing *Wolbachia*

The inhibition of lipid II biosynthesis in *Wolbachia* resulted in an enlarged phenotype indicating the impairment of proper cell division (section 3.1.3). Furthermore the localization pattern of *w*PAL was altered suggesting the interaction of *w*PAL and lipid II. In contrast, after depletion of the cell division protein FtsZ no enlargement or any other aberrant morphological changes could be observed by immunofluorescence as well as transmission electron microscopy (section 3.1.4). To investigate whether FtsZ or *w*PAL are localized at the division sites of *Wolbachia*, *w*Alb B infected insect cells were examined by immunogold staining and transmission electron microscopy. Since the depletion of lipid II by fosfomycin influenced the *w*PAL localization, insect cells treated with fosfomycin were also analyzed. Treated and untreated *w*Alb B infected insect cells were incubated for 9 days and subsequently fixed in paraformaldehyde and glutaraldehyde. Cells were dehydrated in an ethanol series and incubated overnight in a 1:1 mixture of LRWhite resin and ethanol. After two incubation steps in pure LRWhite the resin was polymerized at 37 °C for two weeks. Thin sections were processed by labelling FtsZ and *w*PAL using specific anti-sera (Turner *et al.* 2009; Landmann *et al.* 2012) and a secondary antibody conjugated with 10 nm gold beats. Afterwards the sections were stained using uranyl acetate and lead citrate.

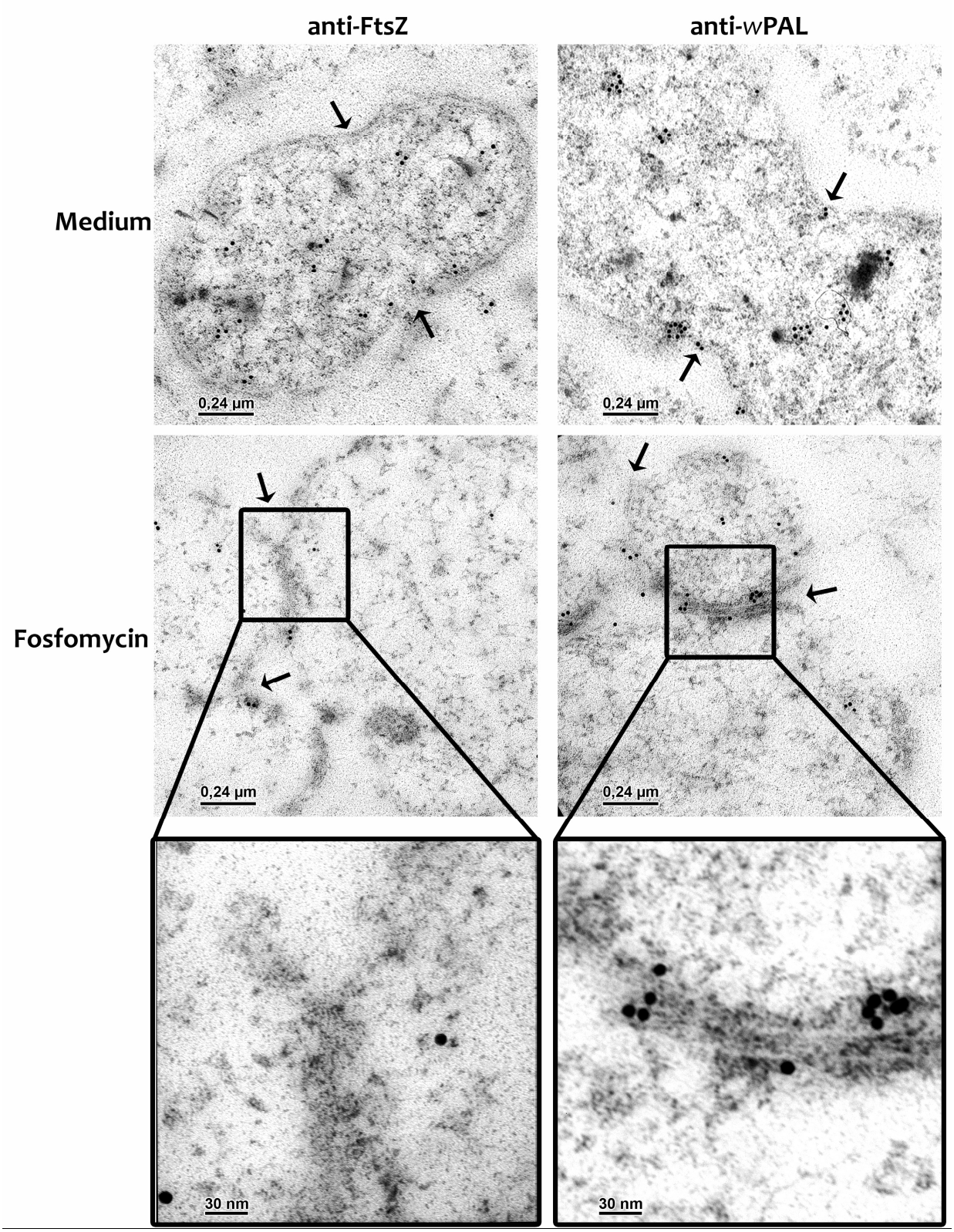


Figure 3.11: Localization of FtsZ and *w*PAL during cell division in fosfomycin treated and untreated *Wolbachia*. C6/36 insect cells infected with *w*Alb B were incubated with or without fosfomycin for 9 days. Cells were fixed in 4 % paraformaldehyde and 0.1 % glutaraldehyde, dehydrated in an ethanol series and incubated in a 1:1 mixture LRWhite : ethanol. Subsequent to two incubation steps in pure LRWhite the resin was polymerized at 37 °C for two weeks. FtsZ and *w*PAL were labelled using FtsZ or *w*PAL anti-serum and a 10 nm gold conjugated secondary antibody. Afterwards thin sections were stained with uranyl acetate and lead citrate. Black boxes mark those areas which were examined in higher magnifications. Arrows depict constriction sites of dividing cells.

Compared to Epon embedded cells (Figure 3.6 and Figure 3.10) the LRWhite embedded cells were less electron dense (Figure 3.11). Between *Wolbachia* and the membrane of their surrounding vacuole free space could be seen. Since LRWhite resin has lower cross-linking than Epon to ensure efficient antigen - antibody binding the structure of LRWhite embedded cells is less conserved. Therefore free space surrounding the *Wolbachia* cells was probably an artefact of the embedding process. In untreated insect cells clearly dividing *Wolbachia* cells were rarely found but after fosfomycin treatment the number of dividing cells was even lower (Table 3.2). As described earlier *Wolbachia* did not form a septum at the division site but seem to separate by binary fission (Wright *et al.* 1978) (Figure 3.11). However, after fosfomycin treatment cell division seemed not only to occur less frequently but exhibited a rather budding-like process separation process in which the separating daughter cell was much smaller in cell size than the mother cell. Additionally, non-separating membrane layers could be observed at the division site resulting in a more persistent attachment of daughter and mother cells (Figure 3.11, lower panels). FtsZ labeling was observed in the cytoplasm of both fosfomycin treated and untreated *Wolbachia* but no FtsZ was found at the cell membranes or at the constriction sites of dividing cells in either case. *w*PAL was located at the *Wolbachia* membranes and in the cytoplasm. In around 50 % of untreated cells *w*PAL labeling could be seen at the constriction sites (Table 3.2). After fosfomycin treatment, in 100 % of cells *w*PAL was found extensively located at the site of daughter cell separation.

Table 3.2: Dividing *Wolbachia* cells analyzed by immunogold staining.

Treatment	Immunogold-Labelling	Number of dividing *Wolbachia* analized	Labelling at constriction sides [%]
Medium	*w*PAL	14	50%
Fosfomycin	*w*PAL	3	100%
Medium	FtsZ	9	0%
Fosfomycin	FtsZ	5	0%

3.2 Characterization of an cell-free *Wolbachia* culture

Since *Wolbachia* are obligate intracellular bacteria their cultivation is very difficult. Only few culture systems exist in which insect cell lines are stably infected with *Wolbachia* strains of arthropods (Fenollar *et al.* 2003; McMeniman *et al.* 2008). Many attempts were made to culture *Wolbachia* outside their host cells and it was shown by Rasgon *et al.* that *Wolbachia* purified from insect cells could be maintained in an insect cell free medium for up to one week but the bacteria were not able to replicate under these conditions (Rasgon *et al.* 2006). Not much is known about the regulation of the *Wolbachia* cell cycle but apparently components of the host are necessary for bacterial growth.

As a first step to establish an insect cell-free culture of replicating *Wolbachia* it was investigated whether *Wolbachia* replication depended on an intact host cell or if a lysate of disrupted host cells is sufficient for *Wolbachia* growth. For this, two different insect cell-free *Wolbachia* suspensions were prepared. The first suspension contained *Wolbachia* purified according to the procedure published by Rasgon *et al.* (Rasgon *et al.* 2006). Briefly, in this procedure insect cells are lysed, *Wolbachia* are pelleted by centrifugation and subsequently suspended in cell culture medium. A second suspension contained *Wolbachia* purified by an abbreviated method in which insect cells are lysed but *Wolbachia* are not pelleted, so that the insect cell lysate remained in the suspension. A 1:5 dilution of each suspension in cell culture medium was incubated in 25 cm^2 cell culture flasks at 26 °C for 7 days. Samples were removed every 2 days and the amount of *Wolbachia* was determined by qPCR using primer specific for the *Wolbachia* 16S rRNA gene. Since *Wolbachia* possess only one 16S rRNA gene copy in their genome, each gene copy equals one *Wolbachia* cell. Gene copy numbers were normalized to the counts at day 0.

In the insect cell lysate containing cell-free *Wolbachia* culture an up to 3-fold increase of 16S rRNA gene copies/µl was observed demonstrating *Wolbachia* replication (Figure 3.12). *Wolbachia* cell numbers increased between day 3 and day 5 and plateaued between day 5 and day 7. In contrast, the amount of *Wolbachia* cultured in cell culture medium decreased from day 0 to day 1 and remained unchanged until day 7.

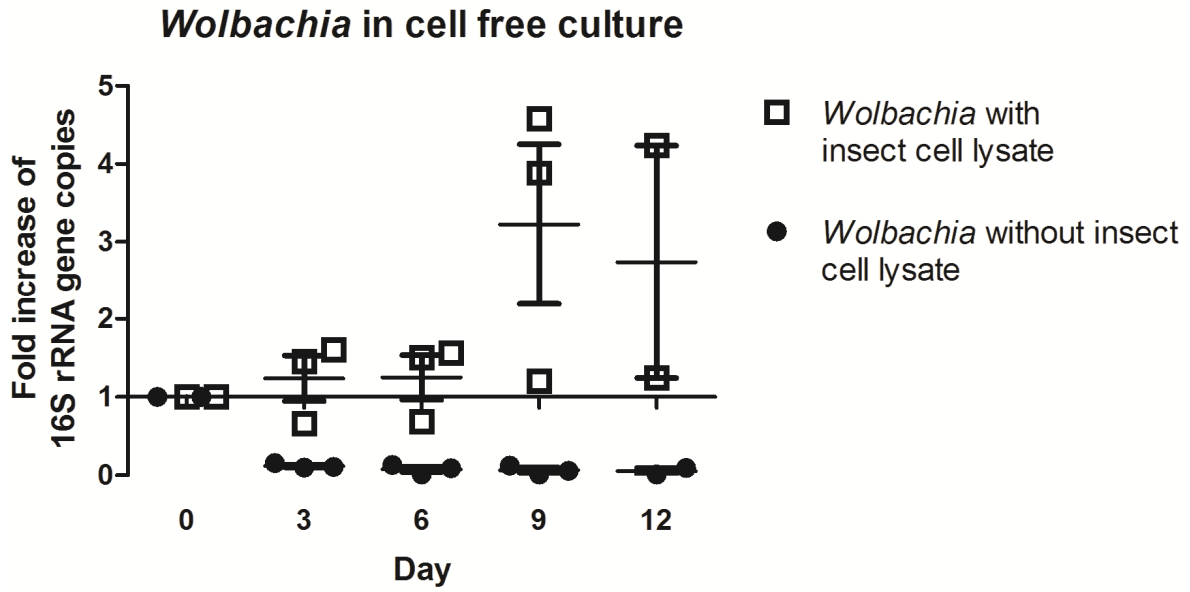


Figure 3.12: Growth curves of *Wolbachia* in insect cell-free culture with and without insect cell lysate. *Wolbachia* were purified from insect cells by the method of Rasgon *et al.* (Rasgon *et al.* 2006) or by an abbreviated method in which the lysate of insect cells maintained in the culture. Cell-free cultures were incubated at 26 °C for 7 days and samples were removed every 2 days. Bacterial quantity was determined by quantifying genomic 16S rRNA gene copy numbers using qPCR and copy numbers were normalized to day 0. The graph is representative for two different experiments. For each time point the mean ± SEM of three samples is shown.

To confirm that *Wolbachia* replication was dependent on the presence of insect cell lysate the bacteria were incubated in cell lysate of *Wolbachia* uninfected C6/36 insect cells prepared according to the abbreviated procedure described above. Additionally, the impact of different dilutions of insect cell lysate on *Wolbachia* growth was investigated in a titration experiment. For this, uninfected insect cells were harvested, counted in a Neubauer counting chamber and lysed. *Wolbachia* were isolated from infected C6/36 insect cells and *Wolbachia* number was determined by qPCR. For each insect cell-free culture, *Wolbachia* were suspended to a final concentration of 0.5 - 1 x 10^3 16S rRNA gene copies/µl in the cell lysate. The final concentration of insect cell lysate was 3.8 x 10^6 uninfected insect cells/ml as calculated from the amount of insect cells counted prior to cell lysis. 1:2 dilutions of this lysate were incubated in a 96-well plate at 26 °C for 12 days and growth was monitored every 3 days by qPCR.

Wolbachia replication was detected in all dilutions of cell lysate prepared from uninfected C6/36 insect cells with the highest copy number of 16S rRNA on day 9 (Figure 3.13). In lysate of 3.8 x 10^6 insect cells *Wolbachia* numbers increased up to 3-fold compared to day 0. In diluted insect cell lysates, the *Wolbachia* replication rate was even

higher, achieving up to a 4-fold increase in lysate of 1.9 x 10^6 insect cells/ml and up to 6-fold increase in lysate of 0.95 x 10^6 insect cells/ml. *Wolbachia* growth proceeded from day 0 to day 9 but *Wolbachia* numbers were decreased on day 12. Based on these results cell lysate prepared from uninfected C6/36 with a final concentration of 0.95 x 10^6 cells/ml was used for further experiments.

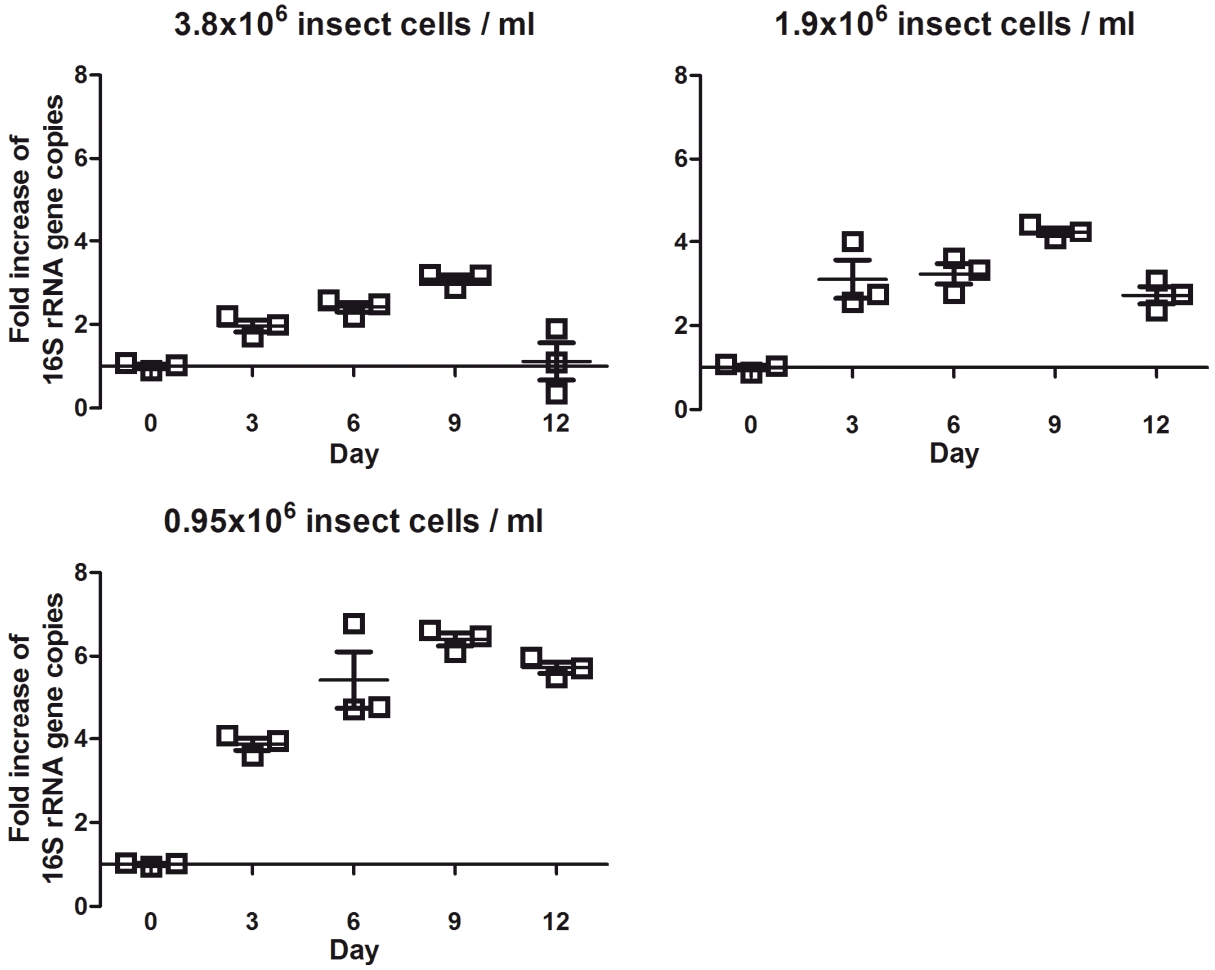


Figure 3.13: Growth curves of *Wolbachia* supplemented with lysate prepared of *Wolbachia* uninfected C6/36 insect cells. Cell lysate of uninfected C6/36 cells was prepared according to the abbreviated procedure. The insect cell number was determined in a Neubauer counting chamber prior to cell lysis. Purified *Wolbachia* were incubated for 12 days in three different dilutions of insect cell lysate with an initial *Wolbachia* concentration of 0.5 – 1 x 10^3 16S rRNA gene copies/µl. Growth was monitored every 3 days by qPCR and data were normalized to day 0. The graph is representative for three different experiments. For every time point the mean ± SEM of three samples is shown.

In the next step the optimal initial concentration of *Wolbachia* for growth in cell-free culture was determined. *Wolbachia* were purified from infected insect cells and lysate of uninfected insect cells was prepared according to the abbreviated protocol. The amount of purified *Wolbachia* was determined by qPCR. Uninfected insect cell numbers were counted in a Neubauer counting chamber prior to cell lysis. Decreasing

concentrations of *Wolbachia* from 10^5 to 10^2 16S rRNA gene copies/µl were suspended in insect cell lysate with a final concentration of 0.95 x 10^6 insect cells/ml as calculated from the amount of insect cells counted prior to lysis. 200 µl *Wolbachia* cultures were incubated in a 96-well plate for 12 days at 26 °C and growth was determined every 3 days by qPCR.

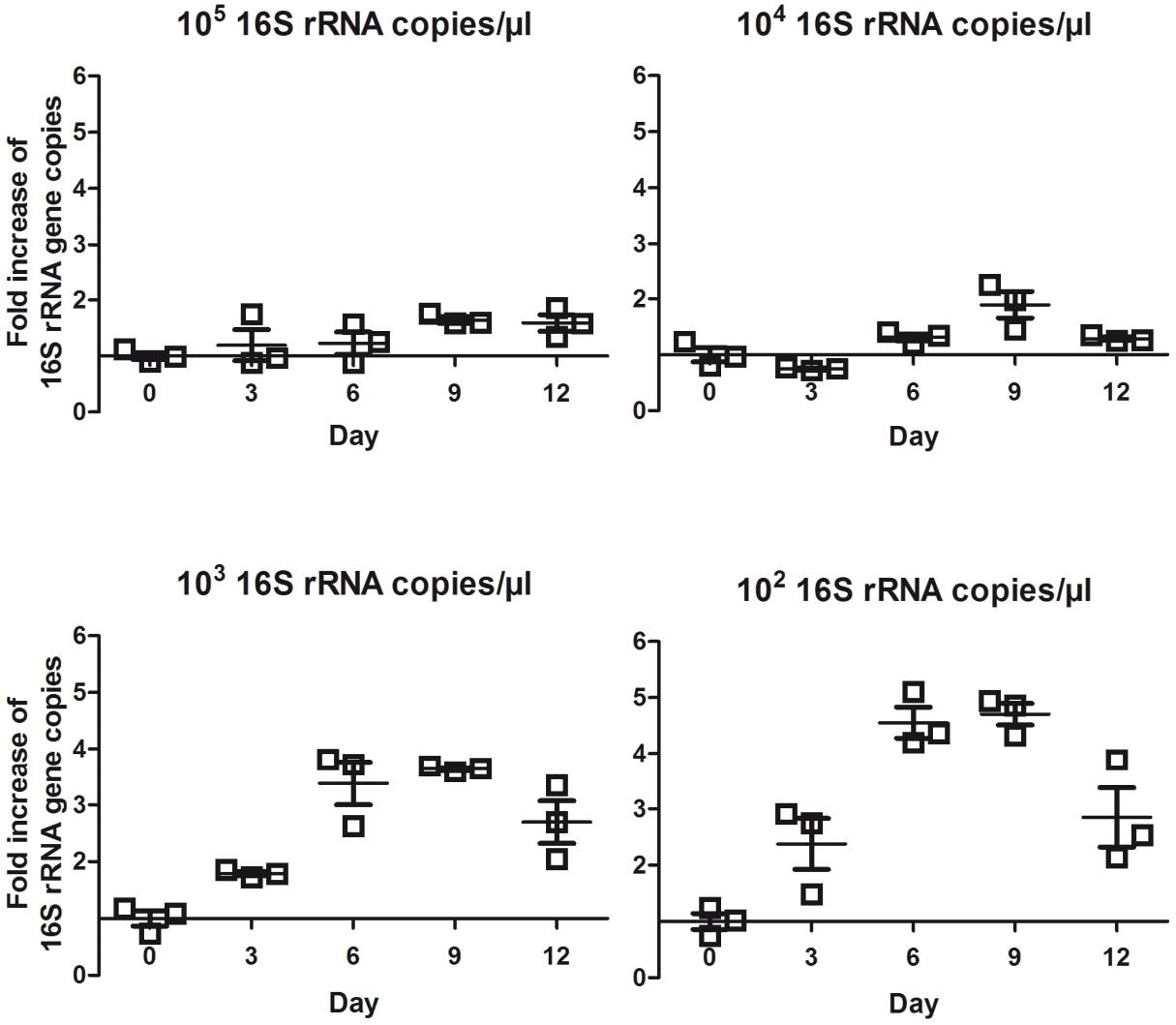


Figure 3.14: Growth curves of different concentrations of *Wolbachia* incubated in cell lysate prepared from uninfected C6/36 cells. Different concentrations of *Wolbachia* were incubated in insect cell lysate with a final concentration of 0.95 x 10^6 uninfected C6/36 cells for 12 days. Growth was monitored every 3 days by qPCR and data were normalized to day 0. The graph is representative for three different experiments. For every time point the mean ± SEM of three samples is shown.

In cell-free culture containing high concentrations of *Wolbachia* of 10^5 to 10^4 16S rRNA gene copies/µl, the *Wolbachia* number slightly increased until day 9 up to 1.5-fold and 1.9-fold, respectively (Figure 3.14). In contrast, in cultures containing lower numbers of *Wolbachia* from 10^3 to 10^2 16S rRNA gene copies/µl, a higher replication rate was

observed between day 0 and day 9 with an increase of up to 3.6-fold and 4.7-fold, respectively. *Wolbachia* numbers were decreased on day 12. Highest *Wolbachia* growth was observed at low initial *Wolbachia* numbers equivalent to 10^2 16S rRNA gene copies/µl. Therefore initial *Wolbachia* concentrations between 10^2 and 10^3 16S rRNA gene copies/µl were used for further experiments.

Subsequent to the optimization of growth conditions of *Wolbachia* in insect cell lysate it was analyzed which components of the insect cell might be necessary for *Wolbachia* growth in an insect cell-free culture. Presumably, *Wolbachia* replication could be dependent on soluble signaling molecules or growth factors provided by the insect cells. As a first step to verify this possibility, insect cell lysate was separated by centrifugation and ultracentrifugation to achieve a rough fractionation of insect cell components (Lodish *et al.* 2000). For this, insect cell lysate prepared from *Wolbachia* uninfected insect cells was centrifuged at 20,000 g for 30 min or ultra-centrifuged at 100,000 g for 60 min, respectively. The supernatant after 20,000 g centrifugation containing microsomes and plasma membranes of the insect cells was retained (Fraction 1) and the respective pellet containing nuclear debris and large cell organelles was suspended in cell culture medium (fraction 2). The supernatant after ultracentrifugation was also retained and contained soluble cytoplasmic contents (fraction 3). Since ultracentrifugation could not be performed under sterile conditions the supernatant was sterile filtered and the pellet was discarded. All three fractions were mixed with purified *Wolbachia* until a final concentration of ~ 10^3 16S rRNA gene copies/µl. The final concentration of insect cells used to prepare the different fractions was 0.95 x 10^6 insect cells/ml as calculated from the of insect cell number counted prior to cell lysis. As a positive control *Wolbachia* were grown in complete insect cell lysate. To determine whether a considerable increase of *Wolbachia* cell number was induced by either of the fractions a negative control was added containing *Wolbachia* cultured in cell culture medium. 200 µl reactions of *Wolbachia* culture were incubated in a 96-well plate for 12 days and growth was determined every 3 days by qPCR.

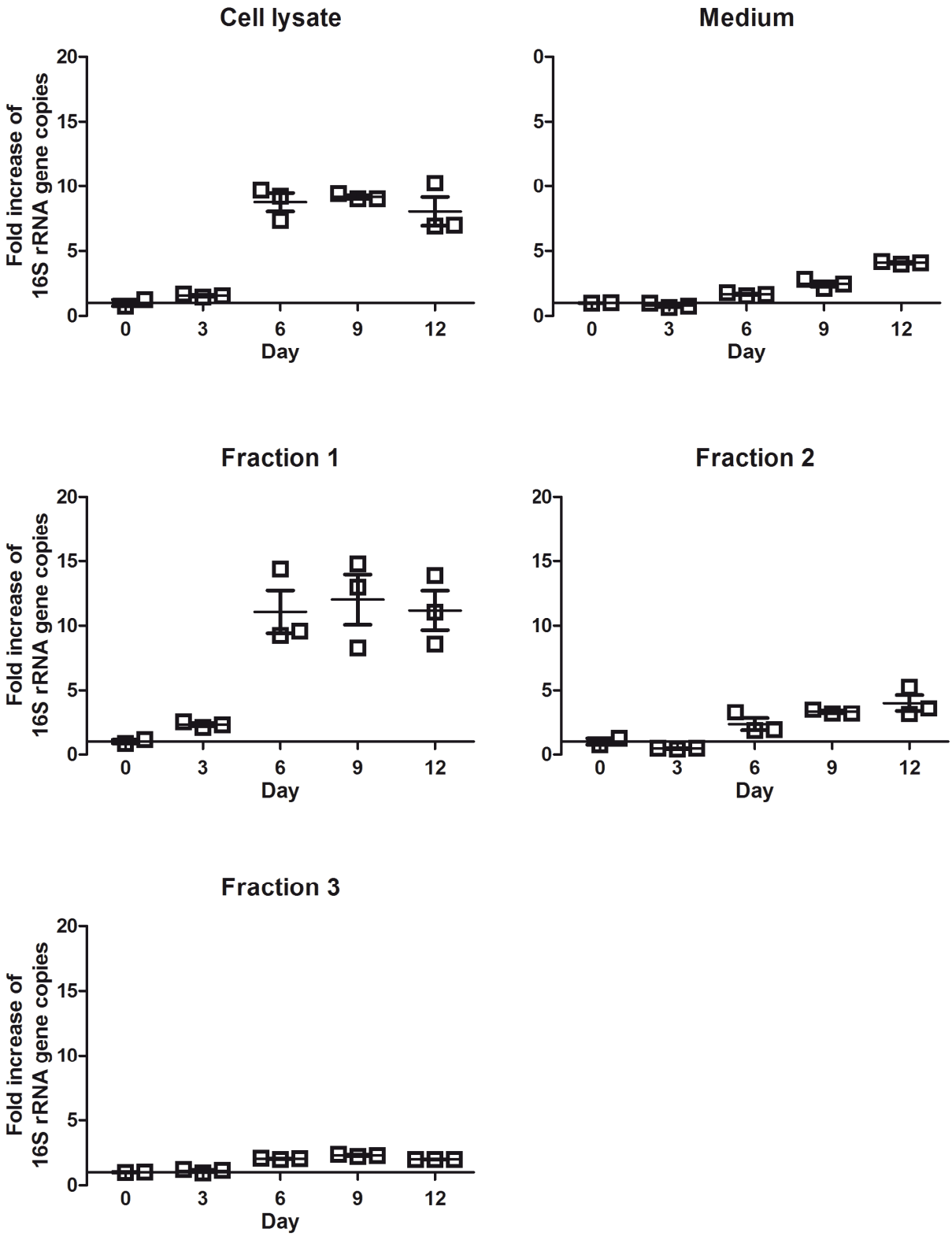


Figure 3.15: Growth curves of *Wolbachia* in insect cell lysate, medium or in different fractions of insect cell lysate separated by centrifugation. A *Wolbachia* suspension and lysate from uninfected C6/36 cells were prepared according to the abbreviated procedure. A portion of cell lysate was fractionated by centrifugation at 20,000 g for 30 min or 100,000 g for 60 min, respectively. *Wolbachia* were incubated in the supernatant retained after 20,000 g centrifugation (Fraction 1), the respective pellet suspended in cell culture medium (Fraction 2) or the supernatant retained after 100,000 g (Fraction 3). Fraction 1 contained microsomes and plasma membranes, Fraction 2 nuclear debris and large organelles and Fraction 3 soluble cytoplasmic content. Growth was compared to reactions containing insect cell lysate or medium, respectively. The initial concentration of *Wolbachia* was ~ 10^3 16S rRNA gene copies/µl. Replication was

monitored by qPCR every 3 days and data were normalized to day 0. The graph is representative for two different experiments. For every time point the mean ± SEM of three samples is shown.

As seen before, *Wolbachia* incubated in insect cell lysate were able to replicate between day 0 and day 9 having a 9-fold increase of 16S rRNA gene copies/µl compared to day 0 (Figure 3.15). *Wolbachia* incubated in cell culture medium showed a slight increase in cell number between day 6 and day 12 up to 4-fold. Comparable to the medium control *Wolbachia* incubated in fraction 2 containing cell organelles and nuclear debris and fraction 3 containing soluble cytoplasmic contents only showed a slight increase in cell number of up to 3-fold (fraction 2) and 2-fold (fraction 3), respectively. However, *Wolbachia* incubated in fraction 1 containing microsomes and plasma membranes replicated in a similar manner to *Wolbachia* incubated in complete insect cell lysate with an apparent higher rate of increase (up to 14-fold on day 9). Therefore for all further experiments *Wolbachia* in cell-free cultures were supplemented with fraction 1 of insect cell lysate that contained microsomes and plasma membranes (membrane fraction).

Growth of C6/36 insect cells in cell culture medium needs the supplementation of FCS to the cell culture medium (Kuno 1983). The exact components of FCS are not known but many hormones, growth factors and nutrients are provided with the serum. Thus, it was elucidated whether FCS is also necessary for *Wolbachia* growth. *Wolbachia* were isolated from infected insect cells and the concentration was determined by qPCR. Two different insect cell lysates were generated, one prepared in cell culture medium supplemented with FCS and the other in cell culture medium without FCS. Nuclear debris and large organelles of insect cells were removed by centrifugation at 20,000 g for 30 min at 4 °C and the supernatant containing microsomes and plasma membranes (membrane fraction) was retained. *Wolbachia* cultures were prepared in the membrane fraction of insect cell lysate with or without FCS, respectively. A negative control of *Wolbachia* incubated in cell culture medium containing FCS was added. The initial concentration of *Wolbachia* was between 2 and 4 x 10^3 16S rRNA gene copies/µl and the membrane fraction was diluted until a final concentration of 0.95 x 10^6 insect cells/ml as calculated from the cell numbers counted prior to cell lysis. 200 µl of *Wolbachia* cultures were incubated in a 96-well plate at 26 °C for 12 days and growth was monitored every 3 days.

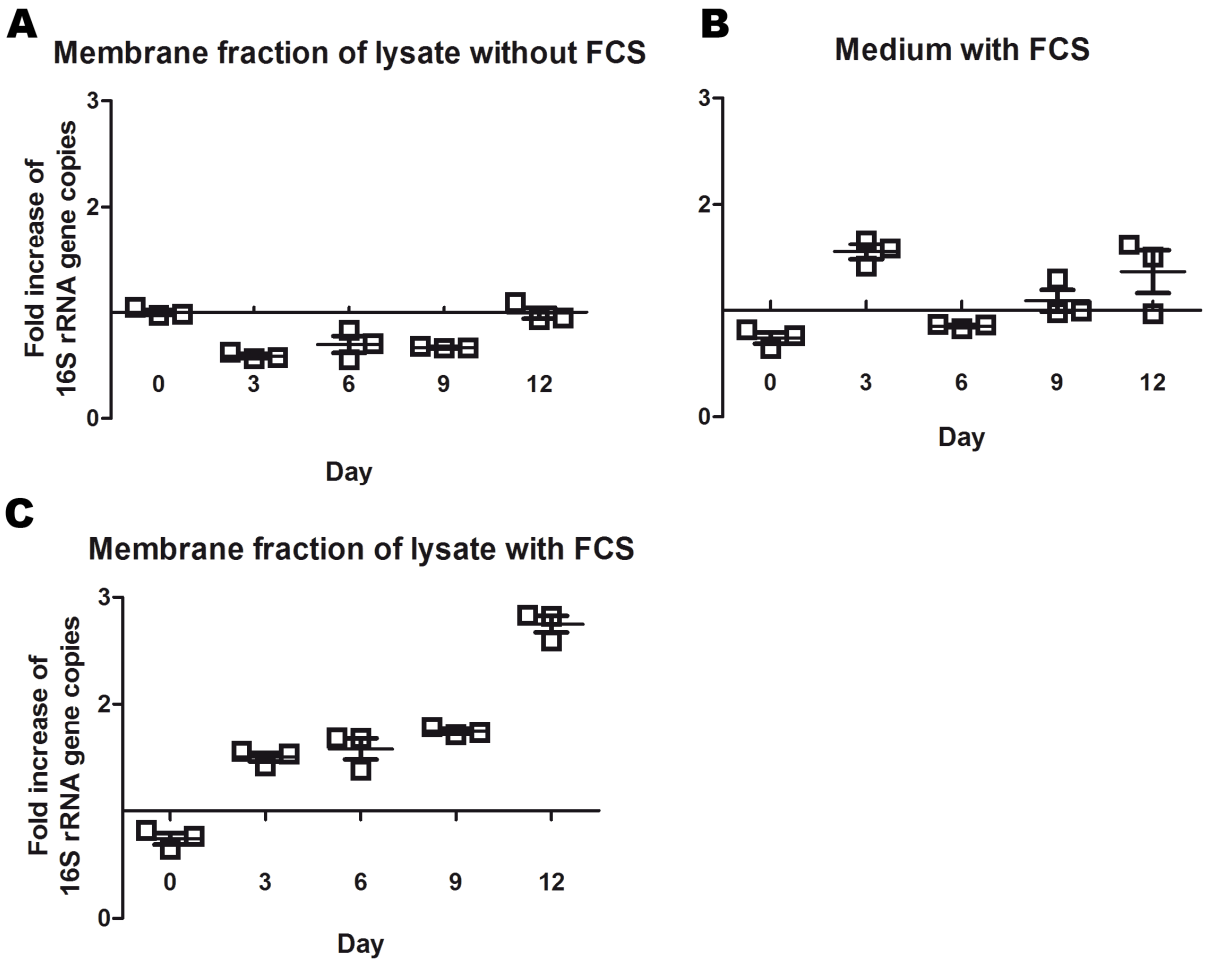
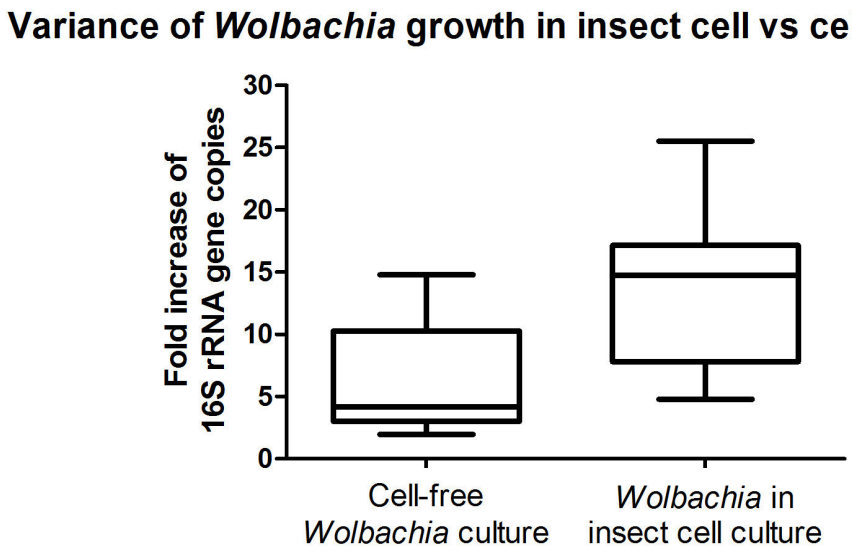


Figure 3.16: Growth curves of *Wolbachia* in insect-cell free culture supplemented with or without FCS. A *Wolbachia* suspension was prepared according to the abbreviated protocol. Two different lysates of uninfected insect cells were prepared in cell culture medium supplemented with or without FCS, respectively. The insect cell lysates were centrifuged at 20,000 g for 30 min at 4 °C and the supernatant containing insect cell membranes and microsomes was retained (membrane fraction). Insect cell-free *Wolbachia* were supplemented with the membrane fraction with or without FCS and cell culture medium, respectively, and incubated at 26 °C for 12 days. The initial concentrations of *Wolbachia* on day 0 were between 2 and 4 x 10^3 16S rRNA gene copies/µl for the respective culture. The final concentration of uninfected insect cells was 0.95 x 10^6 cells/ml as calculated from the cell number counted prior to cell lysis. Replication was monitored by qPCR every 3 days and data were normalized to day 0. Data shown in this graph are representative of two different experiments. For every time point the mean ± SEM of three samples is shown.

Wolbachia grown in the membrane fraction replicated reaching a 2.7-fold increase of 16S rRNA gene copies/µl on day 12 (Figure 3.16 C). In contrast, *Wolbachia* incubated in cell culture medium only slightly replicated up to a 1.5-fold increase on day 3, the copy numbers decreased below 1-fold on day 6 and day 9 and increased again up to 1.5-fold on day 12 (Figure 3.16 B). When supplemented with membrane fraction lacking FCS no increase of *Wolbachia* cell number was detected (Figure 3.16 A).

By supplementation with the membrane fraction of insect cell lysate, replication of *Wolbachia* was induced in insect-cell free *Wolbachia* culture. To determine the stability and growth efficiency of *Wolbachia* in the cell-free culture system growth rates and their variance in this culture system were compared to *Wolbachia* cultured in the C6/36 insect cell line. For each culture system the increase of gDNA 16S rRNA gene copies on day 9 of 10 different experiments were considered. Cell-free *Wolbachia* with an initial concentration between 10^2 and 10^3 16S rRNA gene copies/µl were supplemented with the membrane fraction of insect cell lysate. *Wolbachia* infected C6/36 cells with initial concentrations between 10^3 and 10^4 16S rRNA gene copies/µl were grown in cell culture medium.

In cell-free culture conditions *Wolbachia* cell numbers exhibited a fold increase of between 1.96 and 14.79 in the different experiments with a median of 4.20 and a mean of 6.37 ± 3.97 (Figure 3.17). 25% of all samples were below a 3-fold increase and 75% below a 10.27-fold increase of cell numbers. *Wolbachia* grown in C6/36 insect cells showed higher growth rates between 4.78 and 25.50 fold increase with a median of 14.76 and a mean of 13.57 ± 5.96. 25% of all samples were below a 7.80-fold increase and 75% below a 17.13-fold increase of cell numbers. The standard derivation of the increase of cell numbers in the insect cell culture was marginally lower compared to the cell-free culture system in proportion to the mean values.



	Cell-free *Wolbachia* culture	*Wolbachia* in insect cell culture
Mean +/- standard derivation	6.34 +/- 3.97	13.57 +/- 5.96
Median	4.20	14.76
Minimum	1.96	4.78
Maximum	14.79	25.50
25%-percentile (weighted average)	3.00	7.80
75%-percentile (weighted average)	10.27	17.13

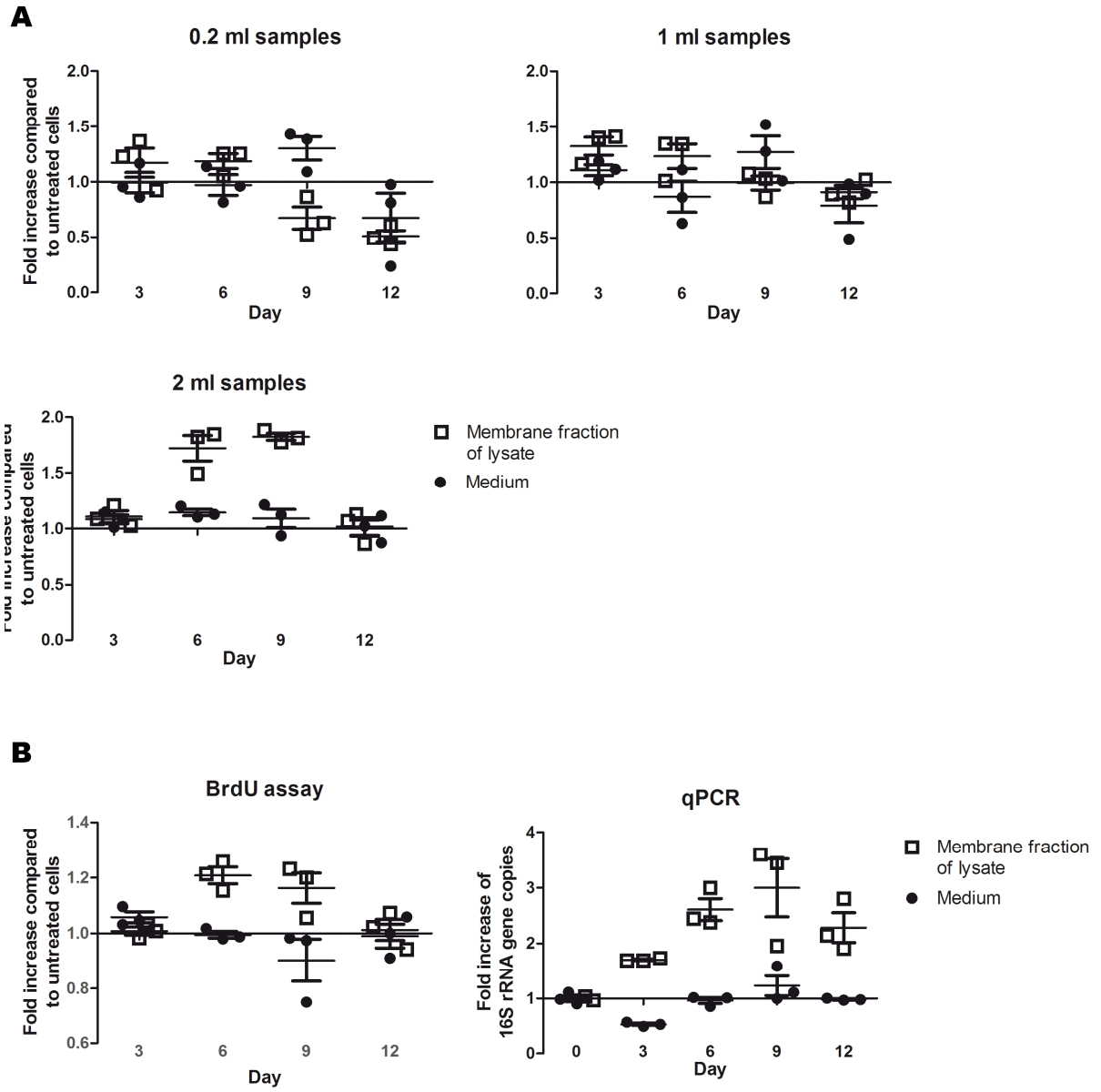
Figure 3.17: Growth variance and efficiency of *Wolbachia* cultured in cell-free compared to insect cell culture. The increase of *Wolbachia* cell numbers grown in cell-free culture or in C6/36 insect cell culture was compared at day 9 combining data from 10 different experiments. Cell-free *Wolbachia* with initial concentrations between 10^2 and 10^3 16S rRNA gene copies/µl were incubated with the membrane fraction of insect cell lysate. *Wolbachia* in insect cells had initial concentrations between 10^3 and 10^4 16S rRNA gene copies/µl. Box plots display the median and extend from the 25% to the 75% percentile. Whiskers represent maximum and minimum values. Descriptive statistic values are depicted in the lower table.

By qPCR, *Wolbachia* replication could be reliably detected in cell free culture in the presence of insect cell lysate. To confirm *Wolbachia* growth as detected by qPCR replication was examined by a distinct method using a BrdU cell proliferation assay. For this, cell-free *Wolbachia* cultures with and without the membrane fraction of insect cell lysate (final concentration of 0.95 x 10^6 uninfected insect cells/ml) were prepared with an initial *Wolbachia* number of 10^3 to 10^4 16S rRNA gene copies/µl. For growth detection using qPCR the gDNA prepared from 0.2 ml culture volumes was used. To evaluate the sample size necessary to detect BrdU incorporation, triplicate *Wolbachia* cultures were grown in 25 cm^2 cell culture flasks for 12 days with and without the addition of BrdU. Every 3 days, *Wolbachia* with culture volumes from 0.2 ml to 2 ml were harvested by centrifugation and fixed in fixation/denaturing buffer. The incorporation of BrdU label in newly synthesized DNA was detected by an ELISA based method using a primary monoclonal BrdU antibody and a peroxidase conjugated secondary antibody. After an

incubation step with the peroxidase substrate tetra-methylbenzidine the reaction was stopped, the samples were transferred into 96-well plates and absorbance was measured at 450 nm. Since the incorporation of BrdU affects the qPCR reaction, samples for qPCR were taken from the BrdU-untreated *Wolbachia* cultures grown in parallel to the BrdU-treated cultures.

Using the BrdU proliferation assay, only low levels of replication of *Wolbachia* in insect cell-free culture could be detected in those samples containing *Wolbachia* incubated in the membrane fraction of insect cell lysate, but at a low level. Of the three different culture volumes used for the BrdU assay only in samples containing *Wolbachia* from 2 ml culture volumes could a considerable fold increase of BrdU incorporation around 1.8 be detected. This growth plateaued on day 6 and day 9 (Figure 3.18 A). Additionally, growth detection using the BrdU proliferation assay was compared to growth detection by qPCR of gDNA (Figure 3.18 B). Using qPCR, continuous *Wolbachia* growth was observed in a culture containing insect cell lysate until a 3 fold increase on day 9 but cell numbers decreased on day 12. For *Wolbachia* cultured in cell culture medium an increase of 1.2 was observed on day 9. In contrast, using the BrdU proliferation assay BrdU incorporation was detected in *Wolbachia* supplemented with insect cell lysate until a 1.2 fold increase on day 6 but then decreased on day 12. In *Wolbachia* cultured in cell culture medium no BrdU incorporation was observed.

Figure 3.18: Detection of *Wolbachia* growth in a BrdU proliferation assay. *Wolbachia* cultures were prepared with an initial concentration of 10^3 to 10^4 16S rRNA gene copies/µl and supplemented with or without the membrane fraction of insect cell lysate (final concentration of 0.95 x 10^6 uninfected insect cells/ml as calculated from the cell number counted prior to cell lysis). BrdU label was added and *Wolbachia* were incubated at 26 °C for 12 days. Every 3 days *Wolbachia* samples were harvested by centrifugation and fixed in fixation/denaturing buffer. Incorporation of BrdU was detected in an ELISA based method using a primary, monoclonal antibody against BrdU and a peroxidase conjugated secondary antibody. Subsequent to incubation with the peroxidase substrate tetra-methylbenzidine samples were transferred into 96-well plates and the absorbance was measured at 450 nm. Data depicted in this graph show the fold increase of incorporated BrdU compared to BrdU-untreated controls. For every time point the mean ± SEM of three samples is shown. **(A)** BrdU assay performed using increasing culture volumes. **(B)** Growth detection by BrdU assay compared to qPCR. For qPCR the DNA of 0.2 ml *Wolbachia* culture was used and data were normalized to day 0. BrdU assay was performed with cells of 2 ml culture volumes. Since incorporated BrdU affects the qPCR the samples quantified by qPCR were taken from the *Wolbachia* culture without BrdU grown in parallel from the same insect cells used to generate the free *Wolbachia*.



Replication of *Wolbachia* in cell-free culture could be elucidated by qPCR and by BrdU cell proliferation assay. Next, the infectivity of *Wolbachia* was investigated by infection of uninfected C6/36 insect cells with *Wolbachia* grown in cell-free culture. A cell-free *Wolbachia* culture with an initial concentration of 0.5 x 10^3 16S rRNA gene copies/µl was prepared from purified *Wolbachia* and the membrane fraction of insect cell lysate (final concentration of 0.95 x 10^6 insect cells/ml). *Wolbachia* were incubated at 26 °C for 12 days in a 75 cm^2 cell culture flask or in a 96-well plate, respectively. Every 3 days triplicate wells of the 96-well plate were harvested for qPCR. After 12 days *Wolbachia* from the cell culture flask were harvested using a cell scraper to suspend sedimented bacteria and 750 µl reactions were applied to uninfected C6/36 insect cells grown in 24-well plates for 3

days. As a negative control *Wolbachia* culture was heated at 95 °C for 10 min prior to infection. After centrifugation at 2,000 g for 1 h at 15 °C the plate was incubated at 26 °C over night. On the next day the liquid was removed and fresh cell culture medium was added containing a 2-fold higher amount of FCS than normally used. After 6 days the insect cells were harvested in fresh culture medium and transferred into culture slides. Additionally samples were taken for qPCR. Insect cells were grown on culture slides for one day. *Wolbachia* infection was subsequently examined by immunofluorescence microscopy using DAPI staining and *w*PAL anti-serum (Turner *et al.* 2009) in combination with an alexa 488 conjugated secondary antibody.

Between day 0 and day 12 the amount of *Wolbachia* increased up to 2.7-fold in cell-free culture supplemented with membrane fraction of insect cell lysate confirming *Wolbachia* replication (Figure 3.19 A). *Wolbachia* from this cell-free culture were used to infect *Wolbachia*-free C6/36 insect cells. 6 days after infection the concentration of *Wolbachia* in the insect cell culture was ~ 140 16S rRNA gene copies/µl (Figure 3.19 B). In contrast, in insect cells infected with *Wolbachia* that were heated prior to infection only 8 16S rRNA gene copies/µl were detected. Immunofluorescence microscopy using a *Wolbachia*-specific antibody confirmed the presence of *Wolbachia* only in the insect cell culture infected with unheated *Wolbachia* (Figure 3.19 C) with *Wolbachia* located near insect cell nuclei. Overall only few *Wolbachia* were found in the insect cell culture.

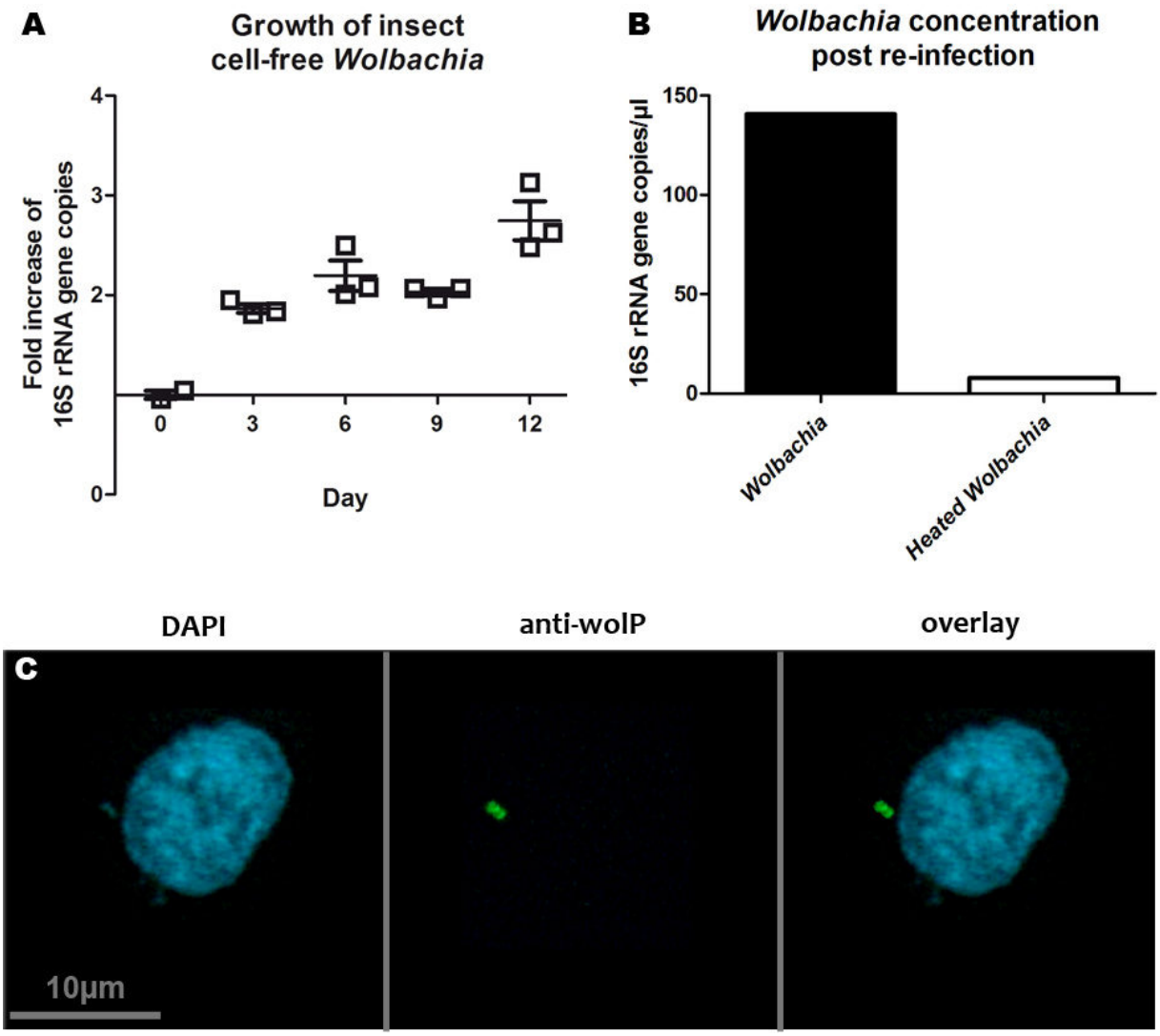


Figure 3.19: Infection of C6/36 cells with *Wolbachia* grown in insect cell-free *Wolbachia* culture. *Wolbachia* were purified and incubated in cell lysate prepared of uninfected insect cells (final concentration 0.95 x 10^6 insect cells/ml as calculated from the amount of insect cells counted prior to lysis) for 12 days at 26 °C in a 75 cm^2 flask. The initial concentration of *Wolbachia* was 0.5 x 10^3 16S rRNA gene copies/µl. On day 12 750 µl reactions of *Wolbachia* culture were added to uninfected C6/36 insect cells grown in a 24-well plate. As a negative control *Wolbachia* culture was heated at 95 °C for 10 min previous addition to insect cells. After centrifugation at 2,000 g for 1 h at 15 °C the plate was incubated over night at 26 °C. On the next day the liquid was removed and fresh cell culture medium containing 10 % FCS was added. Cells harvested in fresh cell culture medium were transferred to culture slides after 6 days. The data shown in this figure are representative of two experiments. **(A)** *Wolbachia* growth in insect cell-free culture was monitored by qPCR every 3 days. For every time point the mean ± SEM of three samples is shown. **(B)** On day 6 after infection, samples were taken for qPCR of insect cells infected with *Wolbachia* and with heated *Wolbachia*, respectively. **(C)** 6 days post infection insect cells were grown on culture slides for 1 day and subsequently examined using immunofluorescence microscopy with *w*PAL anti-serum and an alexa 488 conjugated antibody (green) and with DAPI (blue), respectively.

4 Discussion

4.1 Impact of lipid II and FtsZ on cell division of *Wolbachia*

Endosymbiotic *Wolbachia* are parasitic in arthropods but mutualistic in filarial nematode species (Taylor *et al.* 1999; Fenn *et al.* 2006a). Depletion of *Wolbachia* from the filarial host results in sterility and death of adult worms, which is in contrast to anti-helminthic drugs that only affect microfilaria (Taylor *et al.* 2010; Hoerauf *et al.* 2011). Hence, *Wolbachia* are an effective target for the development of novel antibiotics suitable for mass drug administration. The genomes of obligate intracellular bacteria like *Wolbachia* are characterized by gene reduction and it is hypothesized that those genes maintained in the genome make up the minimal number essential for survival. Although intracellular bacteria do not need a protecting peptidoglycan sacculus *Wolbachia* possess numerous genes involved in lipid II metabolism, yet enzymes that link the lipid II molecules to form the sugar backbone of peptidoglycan are missing (Wu, M. *et al.* 2004; Foster *et al.* 2005). It was proposed that *Wolbachia* might possess a peptidoglycan-like molecule built of peptide cross-links (Foster *et al.* 2005) or that lipid II might be needed for the coordination of cell division (Henrichfreise *et al.* 2009). However, the molecular structure and biological role of *Wolbachia* lipid II are unknown. The investigation of the functional relevance of *Wolbachia* genes involved in lipid II metabolism might not only provide insight into *Wolbachia* biology but might identify novel targets for the development of antibiotics. As part of this PhD project it was shown by RT-PCR that the lipid II metabolism genes that remain in the *Wolbachia* genomes are transcribed in *Wolbachia* strain *w*Alb B (section 3.1.1), supporting a function for these genes in the biology of the endobacteria. These results are in accord with proteomic expression studies of filarial nematodes and their *Wolbachia* endosymbionts in which peptides of lipid II synthesizing enzymes were detected in diverse filarial live cycle stages (Bennuru *et al.* 2011; Darby *et al.* 2012). However, it also was recently discovered that in *Wolbachia* almost 98% of all intact and pseudogenized genes were transcribed, at least on a basal level (Darby *et al.* 2012). Thus, the specific functions of encoded enzymes need to be further characterized on the protein level.

Nevertheless, those genes that are retained and transcribed in *Wolbachia* give first evidence about the composition of the pentapeptide side chain of *Wolbachia* lipid II. *Wolbachia* have lost most genes for amino acid biosynthesis, but have retained the genes for *meso*-DAP biosynthesis (Wu, M. *et al.* 2004; Foster *et al.* 2005). Since the transcription of the *meso*-DAP synthesizing enzymes *dapE* and *dapF* could be confirmed by RT-PCR, it could be excluded that the pathway only possess silent genes. *Meso*-DAP is characteristic for Gram-negative lipid II (Figure 1.1) whereas Gram-positive bacteria commonly have L-lysine (Vollmer, W. *et al.* 2008b). In prokaryotes *meso*-DAP is the direct precursor of L-lysine (Torruella *et al.* 2009), but *Wolbachia* do not encode the gene necessary for the synthesis of lysine from *meso*-DAP (Foster *et al.* 2005). Thus, *Wolbachia* most likely maintained the *meso*-DAP pathway because this amino acid is essential for the synthesis of lipid II and cannot be synthesized and supplied by the eukaryotic host.

D-amino acids are usually found at positions two, four and five of the peptide side chain (Vollmer, W. *et al.* 2008b). Gram-negative bacteria mostly have D-glutamate at position two and the terminal amino acids at position four and five are attached as a dipeptide composed of two D-alanine molecules in almost all prokaryotes. *Wolbachia* express the D-alanine-D-alanine ligase *ddl* but they lack all known amino acid racemases. However, they express genes encoding for the enzymes MetC and GlyA that were shown to have an alternative alanine racemase activity in *E. coli* (Kang *et al.* 2011). Originally, *metC* and *glyA* encode for enzymes of methionine biosynthesis pathway and strikingly are the only two genes of this pathway that are retained in the *Wolbachia* genome. This strongly suggests that they may have an alternative function in these bacteria.

To investigate whether *w*Bm MetC also possess L-alanine racemase activity, the recombinant protein was overexpressed in *E. coli*. Expression of recombinant *w*Bm GlyA was shown to be unfeasible in the *E. coli* expression system although different expression conditions were tested as part of a bachelor thesis carried out in this group (Jülicher 2012). It is known that numerous *Wolbachia* proteins are extremely challenging to recombinantly express (unpublished data, (Foster *et al.* 2009; Schiefer *et al.* 2012, submitted)). Thus, only recombinant *w*Bm MetC was further investigated. The success of expression and purification of recombinant *w*Bm MetC in *E. coli* was verified by SDS-PAGE showing a protein band with the predicted size of *w*Bm MetC in the eluted protein fractions that was not seen in protein lysate from cells containing the empty vector

control. The correlation of this protein band with *w*Bm MetC was previously shown by Western blot using a specific anti-His-tag antibody, thus confirming that the 47 kDa protein band was the recombinant His-tagged *w*Bm MetC (Jülicher 2012). The protein yield was only moderate because expression could not exceed two hours. At later time points of expression the recombinant protein was degraded in the *E. coli* host even at low IPTG concentration and temperatures. In the eluted protein fractions several secondary protein bands beside those of recombinant *w*Bm MetC were observed by SDS-PAGE (Figure 3.3). Most secondary proteins were also found in the empty vector control demonstrating co-purification of native *E. coli* proteins. But purified *w*Bm MetC samples contained an additional protein of 60 kDa size that could not be observed in the empty vector control. Since the size of this protein was larger than *w*Bm MetC it cannot represent a truncated form of the protein due to inaccurate expression and the size of the protein is too small to correspond to protein polymers. Thus, the 60 kDa protein is most probably a co-purified, native *E. coli* protein. Nevertheless, the co-purified protein is larger than known *E. coli* racemases.

In the *in vitro* assay *w*Bm MetC showed L-alanine racemase activity (section 3.1.2) and therefore might provide D-alanine for the terminal dipeptide in the pentapeptide of lipid II. The reaction was dependent on the supplementation of the co-factor PLP indicating a PLP-assisted reaction. In the common bacterial racemases the racemization of L-alanine is achieved by the interaction of PLP and substrate forming an external aldimine intermediate in the active site of the enzyme at which transamination occurs (Griswold *et al.* 2011). Thus, it can be assumed that a similar mechanism takes place in the *Wolbachia* MetC catalyzed reaction. The enzymatic activity appeared to be very low especially compared to the enzyme activity of the recombinant *B. stearothermophilus* racemase used as a positive control. *Wolbachia* exhibit a very long generation time of around 14 h and might therefore possess a rather slow cell metabolism (Fenollar *et al.* 2003). On this account it has been suggested that the kinetic activity of *Wolbachia* enzymes might be generally low. However, it cannot be ruled out that the assay conditions, being non physiological, are less optimal for the activity of the *w*Bm MetC compared to the *B. stearothermophilus* racemase or that additional factors are needed.

The L-alanine racemase activity of *w*Bm MetC was not affected by the compound AVG, an inhibitor of *E. coli* MetC during methionine biosynthesis (Clausen *et al.* 1997),

neither at a concentration of AVG that was shown to effectively inhibit *E. coli* MetC normal enzymatic activity, nor at three times higher concentration. It was demonstrated previously in our group that *w*Bm MetC is still able to catalyze the conversion of L-cystathionine to homocysteine, the reaction that normally takes place during methionine biosynthesis and that is inhibited by AVG. But AVG also did not affect this catalytic activity (Jülicher 2012). This suggests a sterical difference of the active site of *w*Bm MetC compared to the orthologous protein in *E. coli* resulting in the failure of AVG to bind the active site. However, an AVG sensitive, recombinant MetC as a control was not available for the *in vitro* assay to confirm this assumption. In contrast to AVG, the antibiotic D-cycloserine showed an inhibitory effect on *w*Bm MetC *in vitro*. However, high concentrations of D-cycloserine complexes free PLP (Manohar *et al.* 1984) necessary for the *in vitro* activity of *w*Bm MetC. Furthermore, *w*Bm MetC is not an ortholog of racemases that are sensitive to D-cycloserine. Thus, the inhibitory effect of D-cycloserine on *w*Bm MetC is most probably not on account of binding of the inhibitor to the active site of the enzyme but rather due to the depletion of free PLP needed for activity. *Wolbachia* also lack the known racemases that catalyze racemization of L-glutamate to its D-isomer found in the peptide side chain of Gram-negative bacteria. But whether the enzyme MetC or GlyA are able to convert L-glutamate to D-glutamate, which is found at position two, remains to be elucidated.

Commonly, after lipid II is synthesized in the cytoplasm, it is flipped across the cytoplasmic membrane. Each *Wolbachia* strain possesses one gene that encodes a possible lipid II flippase of the SEDS protein family (Table 3.1) and expression of the flippase gene was confirmed in *w*Alb B. Therefore it can be speculated that the lipid II molecule is translocated across the cytoplasmic membrane into the periplasm. The transpeptidase PBP2 and the carboxypeptidase PBP6a are also expressed in *w*Alb B and might participate in the cross-linking of lipid II. In other bacteria, PBP3 together with FtsW and FtsZ are part of the divisome while the elongasome includes PBP2, RodA and MreB (see section 1.1.2). Of every *Wolbachia* strain sequenced so far, only *w*Ri and *w*Mel possess the genes for PBP2 and PBP3, while *w*Bm and *w*Pa possess only the gene for PBP2. Furthermore, every strain exhibits only one potential lipid II flippase, either FtsW or RodA (Table 3.1). Strikingly, *w*Bm possess the division specific *ftsW* gene but the elongation specific *pbp2* gene. This indicates a possible loss of functional differentiation,

if these genes are active in *Wolbachia*, hence displaying that in *Wolbachia* the two currently described multi-enzyme complexes elongasome and divisome (Typas *et al.* 2011) (Figure 1.4) might not be present. Another possibility could be that those enzymes retained possess only one specific function in *Wolbachia*, either they might be necessary for the formation of a cell surrounding macromolecule or for cell division. An exclusive participation in cell division is supported by the fact that enzymes for synthesis and degradation of glycan chains that normally build the backbone of the peptidoglycan macromolecule are lacking (Foster *et al.* 2005; Pfarr *et al.* 2005).

A prerequisite for bacterial cell division is the proper assembly of the divisome and it has been observed that the disturbance of this process results in an aberrant phenotype characterized by filamentation of rod-shaped bacteria or swelling in cocci (Goehring *et al.* 2005; Park, I. S. *et al.* 2005). In *Wolbachia* we observed a similar phenotype subsequent to the blockade of lipid II biosynthesis by fosfomycin at one of the first steps, hence demonstrating that lipid II is essential for the cell division of *Wolbachia*. No aberrant septa or multiple constriction sides could be observed suggesting impairment in an early division phase. In cell wall-less bacteria other than *Wolbachia*, cell division defects were also observed after treatment with cell wall biosynthesis inhibitors. The distantly related, cell wall-less endobacteria *Chlamydia* possess a replicating and a non-replicating developmental stage. Like in *Wolbachia*, many genes of the peptidoglycan biosynthesis, except those for glycosyltransferases, were found in *Chlamydia* but peptidoglycan has never been detected. Nevertheless, *Chlamydia* are sensitive to the PBP inhibitor penicillin, a fact called the chlamydial anomaly (Moulder 1993). It was discovered by electron and video time lapse microscopy that penicillin treatment led to an enlarged morphology of the replicating reticulate bodies while the non-replicating elementary form was not affected suggesting that retained peptidoglycan synthesizing enzymes are needed for cell division (Matsumoto *et al.* 1970; Kramer *et al.* 1971; Skilton *et al.* 2009). The importance of lipid II during cell division has also been shown for organisms with a cell wall. In *E. coli* treatment with cefsulodin, a specific inhibitor of PBP1A and PBP1B, results in osmosensitive *E. coli* L-forms that lack a peptidoglycan sacculus but are viable and able to replicate in a hypertonic medium as seen by the increasing amount of cells (Joseleau-Petit *et al.* 2007). A similar phenotype was observed in *E. coli* mutants lacking PBP1A and PBP1B. It was demonstrated by Joseleau-Petit *et al.* that those L-forms are still able to synthesize

7 % of the normal amount of peptidoglycan. When *E. coli* L-forms additionally were treated with specific inhibitors of PBP2 or PBP3 (amdinocillin and piperacillin / aztreonam, respectively) growth was rapidly blocked and filamentation was observed (Vinella *et al.* 1993; Joseleau-Petit *et al.* 2007). Thus, it was concluded that in *E. coli* peptidoglycan is not only needed to form a stabilizing sacculus but a basal level of peptidoglycan synthesis is essential for cell division. The chloroplasts of the moss *Physcomitrella patens* were shown to be sensitive to several peptidoglycan biosynthesis inhibitors (ampicillin, D-cycloserine, fosfomycin and vancomycin) and treatment with those antibiotics resulted in the formation of macrochloroplasts demonstrating division defects (Katayama *et al.* 2003). In the chloroplast genome genes for lipid II biosynthesis, a transpeptidase and a carboxypeptidase were found and gene disruption or knockout of *Physcomitrella patens* MurE or transpeptidase also led to the formation of macrochloroplasts (Machida *et al.* 2006).

In *Wolbachia,* fosfomycin treatment did not affect membrane integrity as seen by electron microscopy in which no membrane disruptions or leakage of cytoplasm were observed. This is in contrast to the effects seen subsequent to inhibition of peptidoglycan biosynthesis in bacteria that have a cell wall. The ultrastructure of penicillin treated *E. coli* revealed gaps and holes in the peptidoglycan layer that led to leakage of cytoplasmic content (Bayer 1967). In *Pseudomonas aeruginosa* treated with fosfomycin, bleb-like formations on the bacterial surface have been found (Yamada *et al.* 2007). These observations are due to continued peptidoglycan lysis taking place during cell growth (Vollmer, W. *et al.* 2008a) whereas newly synthesized peptidoglycan precursors are absent. Therefore, it can be assumed that the lipid II biosynthesis pathway in *Wolbachia* is conserved because it is essential for cell division but not for membrane stabilization.

Another effect of fosfomycin treatment on *Wolbachia* was a perturbed localization of the lipoprotein *w*PAL suggesting the interaction of *w*PAL with lipid II or its processed form (section 3.1.3). Except for *w*PAL, *Wolbachia* lack petidoglycan binding proteins (Wu, M. *et al.* 2004; Foster *et al.* 2005; Turner *et al.* 2009) that promote the maintenance of the peptidoglycan sacculus in other Gram-negative bacteria (section 1.1.1; (Typas *et al.* 2010)). Commonly, Pal binds specifically to the amino acid *meso*-DAP of the peptide moieties of peptidoglycan and is part of the membrane-spanning Tol-Pal complex (section 1.1.1; (Parsons *et al.* 2006)). This complex was recently demonstrated to be essential for proper

constriction of the outer membrane during cell division in *E. coli* and *C. crescentus* (section 1.1.2; (Gerding *et al.* 2007; Yeh *et al.* 2010). It has been shown by electron microscopy that *Wolbachia* harbor an outer membrane and an inner membrane (Wright *et al.* 1980), although the outer membrane must differ significantly from those of other Gram-negative bacteria since they are unable to synthesize lipid A, a key moiety of lipopolysaccharide (Foster *et al.* 2005). Probably *w*PAL is necessary to connect the two membranes, especially during cell division. However, *Wolbachia* lack the genes encoding the Tol proteins located in the inner membrane. They possess only the gene for TolB which is commonly located in the periplasm and is thought to modulate Pal by competing for peptidoglycan (Gerding *et al.* 2007). Thus, it remains to be elucidated whether *w*PAL has any other interaction partner located in the inner membrane. One possibility might be that during cell division lipid II or a further processed form of the molecule is permanently anchored in the inner membrane by the lipid anchor and therefore connects the inner and outer membranes via the interaction of lipid II with *w*PAL. As seen by immunofluorescence microscopy most *Wolbachia w*PAL is evenly distributed over the cell surface. In contrast, when treated with fosfomycin *w*PAL localizes into a spot-like pattern (Figure 3.5). In *E. coli* it was observed that the Tol-Pal complex was dispersed along the cell envelope in non-constricting cells but accumulates at the division sites in dividing cells (Gerding *et al.* 2007). On account of the long generation time of *Wolbachia* cell division occurs rarely compared to other bacteria and *Wolbachia* size is too small to detect division sites reliably by immunofluorescence microscopy. But subsequent to the treatment with fosfomycin cell division is probably arrested and cell size increases. Low amounts of lipid II are most likely still present in the cell and therefore it can be supposed that bright spots of *w*PAL molecules in treated bacteria might represent *w*PAL bound to residual lipid II or a further processed form of the molecule that are located at early division sites. This is supported by the examination of immunogold labeled *w*PAL in fosfomycin treated and untreated insect cells (section 3.1.5). The immunogold stainings carried out could only be performed with thin sections of cells. Hence, those proteins participating in cell division can only be seen at the edges of constriction sites. In untreated, constricting *Wolbachia* cells *w*PAL was frequently found at the constriction sites located midcell. In fosfomycin treated cells constriction sites were infrequently seen that were delocalized closer to the cell poles resulting in an asymmetrical size of the

constricting cells (Figure 3.11). At such constriction sites multiple membrane layers were seen and several smaller *Wolbachia* cells seemed to be attached to each other indicating an arrested separation process. Considerable *w*PAL labeling was observed at these aberrant constriction sites supporting the hypothesis, as indicated by immunofluorescence staining, that *w*PAL might be localized to arrested early division sites. Interestingly, an asymmetrical constriction process was also seen in penicillin induced aberrant *Chlamydia trachomatis and Chlamydia psittaci*. Removal of penicillin resulted in the recovery of the normal developmental cycle but the aberrant reticulate bodies started to divide asymmetrically in a budding-like process (Matsumoto *et al.* 1970; Skilton *et al.* 2009). Fosfomycin is unstable over time and degradation of the antibiotic might lead to the recovery of cell division in aberrant *Wolbachia* that might occur in a budding-like process as observed in aberrant *Chlamydia*.

Wolbachia are sensitive to antibiotics of the acyldepsipeptide class. ADEPs were shown to induce the dysregulation of the protease ClpP (Brötz-Oesterhelt *et al.* 2005). ClpP facilitates the degradation of misfolded or damaged proteins. The proteolytic core of the protease is formed by the protein ClpP that exhibits the active site inside of a barrel-shaped structure built of tetradecamers. The degradation of proteins is promoted by Clp-ATPases that are located as homohexameric rings around the pores of the barrel. These ATPases unfold to be degraded proteins and introduce them through the pores of the barrel in an ATP-dependent manner (Lee *et al.* 2010). ADEPs compete with the Clp-ATPases for their binding sites and lead to the assembly of the ClpP core and furthermore to the widening of the entrance pores hence resulting in an uncontrolled proteolytic activity (Brötz-Oesterhelt *et al.* 2005). In *Wolbachia* ADEP treatment resulted in the depletion of cell division protein FtsZ (section 3.1.4) (Schiefer *et al.* 2012, submitted). FtsZ is described as one of the most important proteins necessary for cell division of prokaryotes and facilitates the coordinated divisome assembly (section 1.1.2). Consequently, division defects resulting in an aberrant, enlarged phenotype were observed for instance in *Bacillus* containing a *ftsZ* mutation (Park, I. S. *et al.* 2005). It was shown earlier that FtsZ is depleted in strains of *Bacillus subtilis* and *Streptococcus pneumoniae* by treatment with ADEPs leading to filamentation and swelling of the bacterial cells, respectively (Sass *et al.* 2011).

In *Wolbachia* depletion of FtsZ by ADEPs did not result in any obvious aberrant phenotype as observed by immunofluorescence and transmission electron microscopy (section 3.1.4). ADEP treated *Wolbachia* cell size was comparable to those of untreated cells indicating that FtsZ might play a less significant role in cell division of *Wolbachia*. This hypothesis is supported by the finding that *Chlamydia spp.* endobacteria lack any ortholog of the *ftsZ* gene in their genome (Stephens *et al.* 1998). Additionally, the localization of *w*PAL remained unaltered subsequent to ADEP treatment. Immunogold staining of *Wolbachia* FtsZ resulted in labeled spots in the cytoplasm of constricting *Wolbachia* cells but labeling was not observed at sites of constriction suggesting the absence of FtsZ from these spots (section 3.1.5). However, although depletion of FtsZ by ADEPs did not induce an enlargement of the *Wolbachia* cells, as seen after the depletion of lipid II, the bacteria cell numbers were greatly reduced in treated insect cells. Therefore it can be hypothesized that FtsZ is not essential for cell division of *Wolbachia* but might have a different, yet unknown role in these bacteria. This is supported by the fact that *Wolbachia* lack many of FtsZ stabilizing and regulating factors that are essential for proper cell division in bacteria like *E. coli* (Kirkpatrick *et al.* 2011) as shown in Table 4.1. Among division regulating ftsZ inhibitors only the ClpXP genes can be found in *Wolbachia,* while genes for other essential regulators like the MinCD proteins, important for proper positioning of the Z-ring, and the nucleoid occlusion factor SlmA are missing.

Table 4.1: Proteins that promote and regulate Z-rings assembly[1]

Function[2]	Proteine[3]	*w*Pa[4]	*w*Bm[5]	*w*Mel[6]	*w*Ri[7]
Stabilization and membrane tethering	ZapABC[a]	-	-	-	-
	FtsEX[b]	-	-	-	-
	FtsA[c]	YP_001975677.1	YP_197946.1	NP_965983.1	YP_002726734.1
	ZipA[d]	-	-	-	-
Regulators	ClpX[e]	YP_001975341.1	YP_198382.1	NP_966118.1	YP_002727045.1
	ClpP[f]	YP_001975340.1	YP_198383.1	NP_966119.1	YP_002727046.1
	MinCD[g]	-	-	-	-
	SlmA[h]	-	-	-	-
	SulA[i]	-	-	-	-

[1] Accession numbers (as assigned by NCBI) of FtsZ stabilizers and regulators found in all completely sequenced *Wolbachia* strains as elucidated by BLAST alignments.
[2] Effect on FtsZ known to promote or inhibit cell division in Gram-negative bacteria.
[3] Proteins known to be important for stabilization or regulation of FtsZ polymerization in Gram-negative bacteria.
[4] *w*Pa: *Wolbachia pipientis* strain endosymbiont of *C. quinquefaciatus* (NC_010981). BLAST analysis was performed using the genome sequence of the closest related strain *w*Pa (Mavingui *et al.* 2012) since the whole genome sequence of *w*Alb B was not available until the time of investigation.

[5] *w*Bm: *Wolbachia* endosymbiont of *B. malayi* (NC_006833).
[6] *w*Mel: *Wolbachia* endosymbiont of *D. melanogaster* (NC_002978).
[7] *w*Ri: *Wolbachia* endosymbiont of *D. simulans* strain Riverside (NC_012416).
[a] Promote bundling of FtsZ filaments to Z-ring.
[b] Regulate cell wall hydrolysis.
[c] Stabilizes FtsZ bundling and tethers FtsZ to the cell membrane.
[d] Attaches FtsZ to the membrane via a lipid anchor.
[e] Degrade FtsZ protein.
[f] Prevent Z-ring formation at cell poles.
[g] Prevents Z-ring formation over the nucleoid.
[h] Inhibits FtsZ polymerization in case of DNA damage.

However, it was recently demonstrated that expression of *w*Bm FtsZ was upregulated in those filarial developmental stages in which *Wolbachia* replication is observed (Li *et al.* 2011). Furthermore, the FtsZ inhibitor berberine was demonstrated to be active against recombinant *Wolbachia* FtsZ and berberine treatment of *B. malayi* worms resulted in reduced worm motility and reproduction indicating an important role of FtsZ in *Wolbachia*. It was suggested in this study that *Wolbachia* FtsZ is an essential protein of the Wolbachia cell division machinery. But the direct effect of berberine treatment on *Wolbachia* and the resulting phenotype was not investigated and it was not verified whether reduced worm motility was due to the depletion of *Wolbachia* or due to a detrimental effect of berberine on the worm itself. Thus, it needs to be verified whether FtsZ has a nonessential role in cell division of *Wolbachia* while participating in other relevant biological processes and further points have to be considered.

The dysregulation of the Clp protease by ADEPs most likely do not solely result in the degradation of FtsZ but also of other cytoplasmic proteins and it has been shown that the targets of the Clp protease are not conserved among species (Gorbatyuk *et al.* 2005; Sass *et al.*; Raju *et al.* 2012). Recently, Camberg *et al.* provided the first evidence that multiple cell division proteins are probably degraded by ClpXP in *E. coli* (Camberg *et al.* 2011). The lack of phenotypic differences between ADEP treated and untreated *Wolbachia* must therefore not stringently indicate a proper cell division process. If other essential cell cycle regulating proteins of *Wolbachia* are degraded by dysregulated ClpP a consequence might be cell cycle arrest and thus blockade of growth and cell size increase. FtsZ was not detected at constriction sites by immunogold labeling but in thin sections the whole FtsZ ring is not visible and even in *E. coli* only weak labeling of FtsZ at the leading edges of constriction sites has been seen by this technique (Bi *et al.* 1991; Wang *et al.* 1993). It cannot be excluded that the lack of FtsZ labeling at the constriction

sites of *Wolbachia* is artificial. In conclusion, it needs to be further investigated if *Wolbachia* FtsZ participates in cell division or in other essential biological processes.

As mentioned, *Chlamydia spp.* endobacteria lack any gene ortholog of *ftsZ* in their genome (Stephens *et al.* 1998) but interestingly, an antigenic molecule (SEP-antigen) localized as ring-like structure in the dividing stages of *Chlamydia trachomatis and psittaci* was detected (Brown *et al.* 2000). SEP distribution was diffuse in non-dividing stages, indicating a participation of SEP in cell division. The inhibition of the peptidoglycan synthesis resulted in enlarged *Chlamydia* cells, in which SEP was distributed at distinct sites. The chemical nature of the SEP molecule is still unidentified but was hypothesized to be nonproteinaceous. *Chlamydia* and *Wolbachia* are characterized by their intracellular life-style and exhibit similar cell division mechanisms (Henrichfreise *et al.* 2009). Strikingly, after the inhibition of peptidoglycan synthesis the re-distribution of SEP in *Chlamydia* and of *w*PAL in *Wolbachia* show a similar pattern. Since the *w*PAL distribution seems to reflect the localization of lipid II in *Wolbachia* it can be proposed that the nonproteinaceous SEP antigen might be lipid II or a further processed form of the molecule. As a consequence, lipid II might not only play an essential role in the cell division of *Wolbachia* as a monomer, but might even build a ring-like structure formed of peptide cross-links at the division site as part of the divisome. Since FtsZ is absent from *Chlamydia* and the results presented in this thesis indicate that it does not participate in cell division of *Wolbachia* it is proposed that a ring-like structure built of cross-linked lipid II interacting with *w*PAL might be involved in the constriction process in these bacterial species (Figure 4.1).

However, the mechanical force necessary for constriction of the inner membrane at the septum is normally obtained from the GTPase activity of FtsZ and, for outer membrane constriction via Tol-Pal, from TolA that is energized by an ion potential over the inner membrane. Additionally, the recruitment of divisome proteins and proper positioning of the division site are achieved by FtsZ and FtsZ regulating factors (Gerding *et al.* 2007; Kirkpatrick *et al.* 2011). In *Chlamydia* it was proposed that the cytoskeletal protein MreB might compensate the lack of FtsZ in these bacteria (Gaballah *et al.* 2011). The biological function of MreB in *Wobachia* has not been elucidated, but a participation in cell division might be possible. Therefore it remains to be investigated whether a ring-like structure of lipid II molecules essential for the division of *Wolbachia* and *Chlamydia*

exists and how the assembly of the divisome as well as the generation of mechanical force necessary for constriction is facilitated.

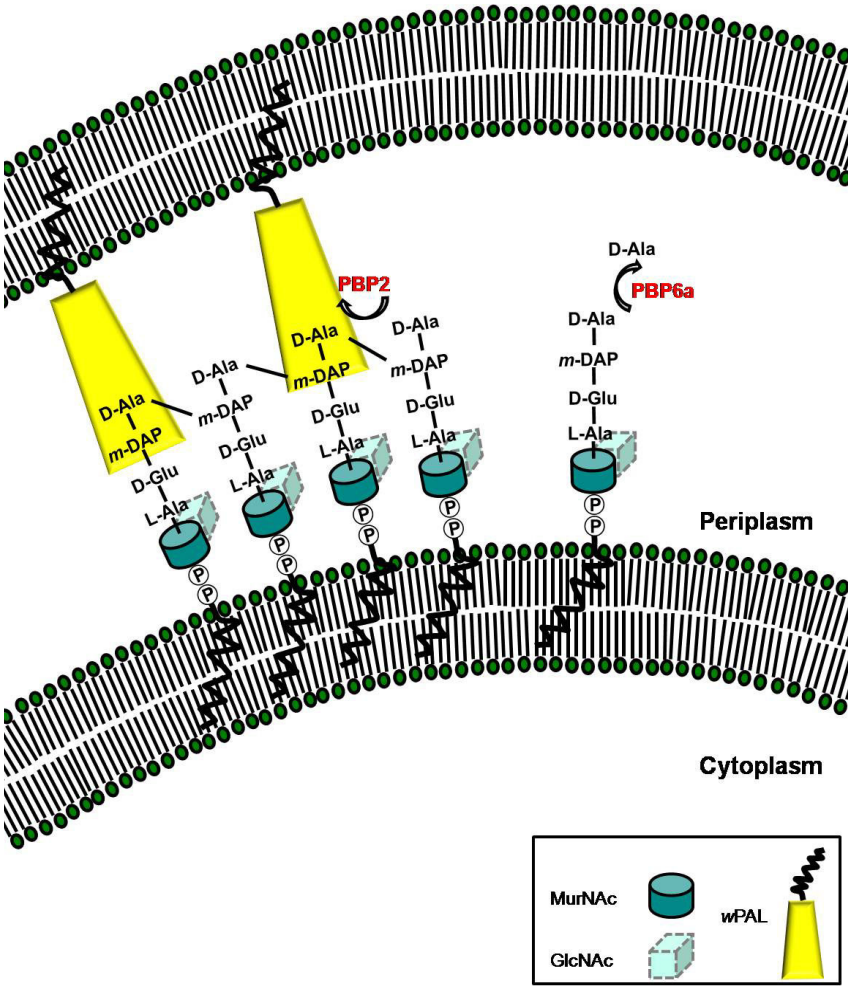


Figure 4.1: Model of a macromolecule built of cross-linked lipid II at a constriction site of a dividing *Wolbachia* cell. Lipid II monomers are cross-linked by the action of the carboxypeptidase PBP6a and the transpeptidase PBP2 to form a ring-like structure involved in cell division. The macromolecule could be linked to the outer membrane by the binding of *w*PAL which might be necessary for constriction of the outer membrane.

4.2 Growth requirements of *Wolbachia* in a cell-free culture

In vitro culture systems of *Wolbachia* necessary for the exploration of the *Wolbachia* biology are few and only *Wolbachia* strains naturally occurring in arthropods have been successfully cultured in insect cell lines (Fenollar *et al.* 2003; McMeniman *et al.* 2008), while all attempts to culture *Wolbachia* of filarial nematodes have failed (McNulty *et al.* 2010). The elucidation of the endobacteria maintained in insect cell lines is extremely challenging because they are protected from the environment by four lipid membrane

layers: the insect cell membrane, the membrane of the vacuole which encloses the *Wolbachia* in the cytoplasm of the insect cell and the bacterial outer and inner membranes. Hence, molecular biological techniques are mostly impossible to apply to *Wolbachia* cultured in insect cell lines, e.g. treatment of *Wolbachia* with antibiotics like vancomycin that are too large to pass the membranes. In an extracellular culture system treatment with large molecules would be possible and could be used to elucidate *Wolbachia* biology as well as to provide a starting point for the development of small, effective antibiotics sensitive against *Wolbachia*. Rasgon *et al.* had shown in 2006 that *Wolbachia* purified from insect cells could be maintained in cell-free culture medium for up to one week without loss of viability or infectivity. However, *Wolbachia* in this culture did not replicate outside of the insect cell (Rasgon *et al.* 2006).

In this PhD project it was demonstrated that *Wolbachia* were not only viable when maintained in a cell-free culture, but underwent replication when lysate from uninfected insect cells was added to the medium as detected by qPCR. Furthermore the incorporation of BrdU into the DNA of replicating *Wolbachia* could be detected in cell-free culture confirming *Wolbachia* growth. However, the BrdU proliferation assay was less sensitive compared to qPCR and 10x higher *Wolbachia* numbers were needed to demonstrate *Wolbachia* growth. Furthermore considerable growth beyond day 6 was only detected by qPCR whereas the detection by BrdU incorporation plateaued between day 6 and day 9. It has been previously shown that the BrdU molecule is stable in cell culture at least for 1 month but after removal of BrdU from the culture medium of eukaryotic cells a rapid decrease of incorporated BrdU was observed (Sauerzweig *et al.* 2009). This decrease was partially due to DNA replication during cell division in the absence of BrdU. But it was also observed that BrdU was transferred into the cytosol probably as a consequence of DNA repair mechanisms. Although the BrdU label is not removed from the cell-free *Wolbachia* culture it cannot be excluded that BrdU is displaced from the DNA over time or that it has a detrimental effect on *Wolbachia* replication.

Viability and infectivity of *Wolbachia* from a 12 day old cell-free *Wolbachia* culture was demonstrated by infecting uninfected C6/36 insect cells, with *Wolbachia* DNA detectable 6 days post infection. Furthermore, by immunofluorescence microscopy *Wolbachia* were found in C6/36 insect cells infected with bacteria and were located near insect cell nuclei indicating their localization in the insect cell cytoplasm. The infection

rate was very low with few insect cells containing few *Wolbachia*. However, compared to the infection experiments of Rasgon *et al.* a lower *Wolbachia* concentration was used in the cell-free culture since concentrations were shown to be beneficial for cell-free growth. Therefore the *Wolbachia* cell number per insect cell used for infection (14 bacteria/cell) was significantly lower than that used in the previously published infection experiments (2,600 bacteria/cell) (Rasgon *et al.* 2006) hence resulting in lower infection rates. When *Wolbachia* were killed by heating prior to infection, only very low amounts of DNA were detected in the insect cells 6 days post infection. By immunofluorescence microscopy, no *Wolbachia* were found in this cell culture. Thus, it is likely that the DNA detected in the insect cell culture infected with heated *Wolbachia* does not represent viable *Wolbachia* but is residual *Wolbachia* DNA from the heated, dead *Wolbachia* that were added to the cell culture. In conclusion, these results indicate that *Wolbachia* from insect cell-free culture replicate and are viable and infective after 12 days.

Wolbachia growth in insect cell-free culture was dependent on the initial *Wolbachia* concentrations with higher concentrations resulting in lower levels of replication. A reason might be an insufficient supply of nutrients. Although *Wolbachia* are sensitive to fosfomycin, an antibiotic that is taken up by glucose-6-phosphate and glucose-3-phosphate transporters in other bacteria (Takahata *et al.* 2010), to date no genes for known sugar transporters have been described in the *Wolbachia* genome (Foster *et al.* 2005). Hence, it was proposed that *Wolbachia* metabolize pyruvate and intermediates of the tricarboxylic acid cycle derived from amino acids (Foster *et al.* 2005). In the insect cell-free *Wolbachia* culture essential and nonessential amino acids are provided in excess by the cell culture medium as well as pyruvate and sugars. *Wolbachia* replicate slowly in the culture and a competition for nutrients is unlikely. Another possibility might be that *Wolbachia* densities are regulated by a yet unknown, intrinsic or host cell derived mechanism. This possibility might explain the observation that *Wolbachia* cell numbers inside insect cells do not reach a density that would negatively affect the survival of the insect cell. But how *Wolbachia* growth is regulated remains to be elucidated.

In contrast, it was observed that increasing the amount of uninfected insect cells used to prepare insect cell lysate had a detrimental effect on *Wolbachia* growth rather than increasing replication. However, *Wolbachia* replication inside their host cells is a complex and tightly regulated process (McGraw *et al.* 2002; Ruang-areerate *et al.* 2004).

The C6/36 insect cell culture was originally generated from *A. albopictus* larvae and therefore consists of cells in different cell cycle stages and of different cell types (Singh 1967; Igarashi 1978). Hence, it has to be considered that *Wolbachia* growth inhibiting factors present in a subset of insect cells might accumulate when high amounts of insect cells are used for lysate preparation. The potential inhibitory impact of components of the insect cells is supported by the fact that *Wolbachia* cell numbers incubated with the membrane fraction of insect cell lysate increased more than *Wolbachia* incubated in unfractionated lysate.

This indicates that inhibitory components might be removed by the fractionation and might remain in the lysate fraction containing large organelles and nuclear debris. But whether any inhibitory component can be found in this fraction remains to be validated. The membrane fraction of insect cell lysate induced *Wolbachia* growth but no replication occurred when the cytoplasmic fraction of the insect cells was used to supplement the medium. This membrane fraction mainly contained microsomes derived from internal membranes like the endoplasmic reticulum and cytoplasmic membranes. Notably, it has been shown in 2003 by Lin and Rikihisa that the survival of endobacteria of the species *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* that are closely related to *Wolbachia spp.* is dependent on the incorporation of cholesterol derived from their host cell (Lin *et al.* 2003). The depletion of cholesterol from the endobacteria resulted in disturbances of membrane integrity. Similar to *Wolbachia spp.*, *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack most genes for peptidoglycan synthesis and cannot produce lipid A, therefore the authors proposed that in *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* cholesterol might be necessary to promote membrane stability that cannot be facilitated by peptidoglycan as in other bacteria. Furthermore it was found that infectivity of these bacteria was essentially dependent on the presence of cholesterol in their membranes. The cell membranes of *Chlamydia trachomatis* also contain cholesterol obtained from the host cell and indications were gained that both, *de novo* synthesized and low-density lipoprotein derived cholesterol, are transported to the *Chlamydia trachomatis* via the Golgi apparatus (Carabeo *et al.* 2003). In fly embryos infected with *Wolbachia pipientis* it has been demonstrated that the vacuole surrounding *Wolbachia* inside the cytoplasm of the host cell are Golgi-related vesicles that are concentrated near the site of membrane biogenesis (Cho *et al.* 2011) and that are in contact with cisterns of

the endoplasmatic reticulum (Popov *et al.* 1998). Although insect cells are cholesterol auxotrophs they assimilate cholesterol from their environment, as well as sterols that they are able to convert to cholesterol, that is then incorporated in the plasma membrane and also in internal membranes like those from the Golgi apparatus (Rolls *et al.* 1997). As investigated in sections 3.1 and 4.1 *Wolbachia* might need the peptidoglycan precursor lipid II for cell division rather than for membrane stabilization. Hence, it can be speculated that *Wolbachia* might incorporate cholesterol derived from the Golgi apparatus of their host cell to promote membrane stability. Consequently, for *Wolbachia* in insect cell-free culture, cholesterol might be a limiting factor and supplementation with the membrane fraction of an insect cell lysate might therefore be necessary to induce growth. Beside cholesterol, sphingomyelin, a sphingolipid that is also exclusively synthesized by eukaryotes, was found in membranes of *Chlamydia trachomatis* but its role in *Chlamydia* biology is unknown *(Carabeo et al. 2003)*. In contrast, in *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*, an uptake of sphingomyelin has never been detected (Lin *et al.* 2003). Insects do not have sphingomyelin but contain ceramide phosphorylethanolamine instead (Luukkonen *et al.* 1973). This sphingolipid might be taken up by *Wolbachia* from their host membranes and could also be limiting for their growth.

Nevertheless, components of the membrane fraction like cholesterol cannot be the only necessary factor for *Wolbachia* growth outside their host cell since *Wolbachia* were not able to grow in insect cell-free culture supplemented with the membrane fraction of lysate when FCS was lacking. The composition of FCS is unknown but it is very likely that, similar to eukaryotic cells in cell culture, the serum provides proteins, carbohydrates, lipids, vitamins and other factors essential for *Wolbachia* viability and replication.

The variance of *Wolbachia* replication rates in cell-free culture between different experiments was similar to those of *Wolbachia* cultured inside insect cells. In both culture systems only a weak statistic normal distribution was observed reflecting growth variability occurring over time that might originate from variances of temperature, medium or cell culture passage. However, the mean growth rate of *Wolbachia* in cell-free culture is about 3.5 times lower compared to *Wolbachia* cultured in insect cells. This indicates that beside the need of FCS and membrane fraction of insect lysate for cell-free *Wolbachia* culture other constituents seem to be missing.

The mechanism of *Wolbachia* replication is, despite intense investigation, still unclear but apparently the nature of both host and *Wolbachia* seem to have an impact on the complex regulation. It has been previously shown that replication of *Wolbachia* is influenced by the host species as well as by tissue-specific differences. When *Wolbachia* of the strain *popcorn* from *Drosophila menalogaster* were transferred into *Drosophila simulans* an increase of *Wolbachia* density was observed demonstrating the impact of the host on *Wolbachia* replication. Furthermore *Wolbachia* replication differed between heads and ovaries indicating a tissue-specific control mechanism (McGraw *et al.* 2002). *Wolbachia* replication is also associated with the stage of the host life cycle. In *Drosophila melanogaster* infected with *Wolbachia popcorn* the endobacteria rarely replicate during fly development, but start to proliferate massively in the adults (Min *et al.* 1997). In *A. albopictus* replication completely stops during the diapause of mosquito eggs in which no host cell division occurs demonstrating the dependence of *Wolbachia* cell division on host cell replication (Ruang-areerate *et al.* 2004). In the filarial nematode *B. malayi Wolbachia* numbers do not greatly increase in microfilaria to infective-stage larva within the mosquitoes, but proliferate greatly after infection of a vertebrate host (Landmann *et al.* 2012). At the molecular level, a mechanism of interaction between host and *Wolbachia* has been recently described in *Aedes agypti* in which *Wolbachia* densities were dependent on host miRNAs (Hussain *et al.* 2011).

Altogether these findings provide insight into the complexity of *Wolbachia* replication that influences the cultivation of the bacteria in cell-free culture. This is probably associated with the differences in cell types and cell cycle stages of the insect cells used to generate lysate. In some experiments a slight increase of *Wolbachia* numbers cultured in cell culture medium without insect cell lysate was detected while in most cases no growth was observed. One possibility might be that in the *Wolbachia* suspension generated from infected insect cells sufficient components that can induce a weak replication rate are present.

Further elucidation of this culture system will be necessary to optimize *Wolbachia* growth outside their host cells and to gain insight into the multiple mechanisms that influence and regulate replication. To determine nutrient requirements for cell-free growth of *Wolbachia* the differences in gene expression of *Wolbachia* cultured with and without insect cells could be investigated. By such an approach a complex medium was

designed for the cell-free growth of the obligate endobacteria *Coxiella burnetii* based on expression microarrays, genomic reconstruction and metabolite typing (Omsland *et al.* 2009). Notably, distinct pathogenic *Coxiella burnetii* strains showed different growth efficiencies in the designed medium (Kersh *et al.* 2011) indicating that nutrient requirements for endobacteria might be strongly species and strain specific. Different to *Wolbachia*, *Coxiella* exhibit a less symbiotic interaction with the host cell and can even persist in the extracellular environment (Heinzen *et al.* 1999). Thus, *Coxiella* might be less influenced by the life cycle of the host cell, a factor that could be more important for *Wolbachia* replication.

Nevertheless, the establishment of this culture system represents a further step in the effort to cultivate *Wolbachia* extracellularly and might also give important cues for the extracellular cultivation of other endobacteria. Moreover a powerful tool for the exploration of *Wolbachia* biology and *Wolbachia*-host interactions is provided by *Wolbachia* cultivated in an insect cell-free *in vitro* system.

5 Summary

The characterization of *Wolbachia* endobacteria is of urgent need for a better understanding of *Wolbachia* biology and for the identification of new drug targets for anti-filarial therapy. In this PhD project important insights into structure and function of the peptidoglycan precursor lipid II in *Wolbachia* were gained. It was shown that the *Wolbachia* genes of lipid II metabolism are expressed on the RNA level and a lipid II pentapeptide containing *meso*-DAP and a terminal D-alanine dipeptide is predicted. The occurrence of D-alanine was supported by the identification of secondary enzyme activity, that of L-alanine racemase, by *Wolbachia* MetC that may compensate the lack of known amino acid racemases. Strikingly, inhibition of lipid II synthesis by fosfomycin resulted in enlarged *Wolbachia* cells indicating a cell division defect, whereas membrane integrity was not affected. Thus it is proposed that lipid II in *Wolbachia* is essential for cell division but not for membrane stabilization. Fosfomycin treatment also resulted in redistribution of *w*PAL lipoprotein suggesting an interaction of *w*PAL and lipid II and a participation of *w*PAL in cell division, as seen for other bacterial species. Interestingly, depletion of cell division protein FtsZ by ADEPs did not result in cell enlargement and with immunogold labeling FtsZ was not detectable at *Wolbachia* constriction sites. This might hint at a nonessential role of FtsZ in the cell division of *Wolbachia.* However, the function of FtsZ in *Wolbachia* must be further elucidated.

In the second part of this PhD project the culturing of *Wolbachia* in a host cell-free *in vitro* system was established, providing a powerful tool for molecular biological applications. By supplementation of medium with insect cell lysate, *Wolbachia* replication could be achieved. The membrane fraction of the insect cell lysate was shown to be essential for growth and, as indicated from other endobacteria, cholesterol might be a promising candidate as essential factor that has to be further investigated. However, multiple factors most likely play a role in inducing and regulating *Wolbachia* replication outside their host cell since growth was also dependent on the presence of FCS and replication rates in insect cell-free culture was lower compared to *Wolbachia* grown inside insect cells. Therefore the investigation of these growth facilitating and regulating factors is necessary for the further optimization of this host cell-free culture system.

6 Appendix

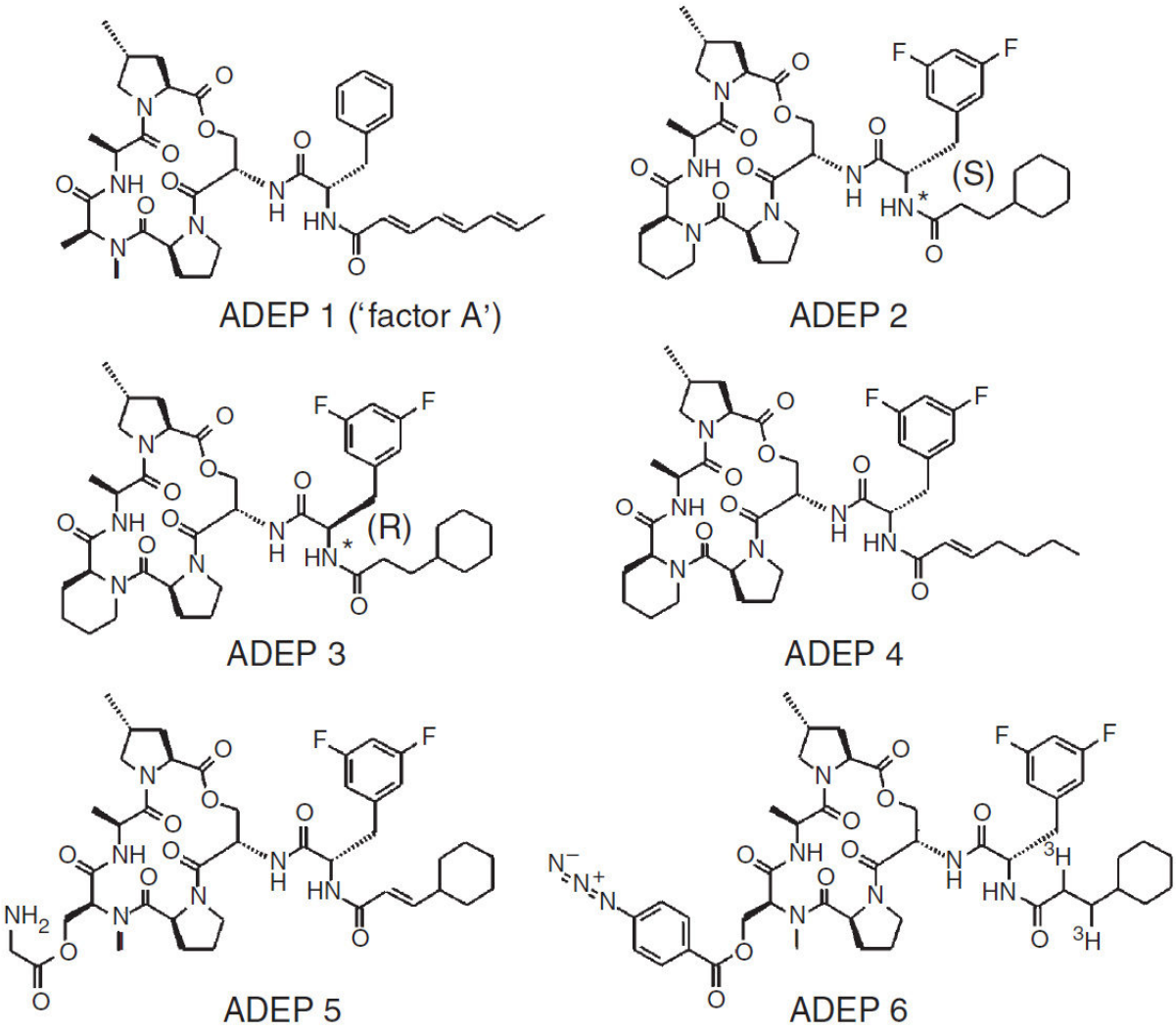


Figure 6.1: Molecular structures of acyldepsipeptide derivatives (Brötz-Oesterhelt *et al.* 2005). ADEP 1 ('factor A') is the natural product of *Streptococcus hawaiiensis* NRRL 15010. ADEP 2 and ADEP 4 are optimized congeners of ADEP 1. ADEP 3 differs from ADEP 2 only by the conformation of the difluorophenylalanine side chain (stereocenter indicated by an asterisk) but is antibacterially inactive. In ADEP 5 a NH_2 functionality is introduced in its southwestern region that allows coupling to NHS-activated Sepharose. ADEP 6 carries a tritium label and an arylazide moiety for crosslinking studies.

7 Literature

Bayer, M. E. (1967). "The cell wall of *Escherichia coli*: early effects of penicillin treatment and deprivation of diaminopimelic acid." J Gen Microbiol 46(2): 237-246.

Bennuru, S., Z. Meng, J. M. Ribeiro, R. T. Semnani, E. Ghedin, K. Chan, D. A. Lucas, T. D. Veenstra and T. B. Nutman (2011). "Stage-specific proteomic expression patterns of the human filarial parasite *Brugia malayi* and its endosymbiont *Wolbachia*." Proc Natl Acad Sci U S A 108(23): 9649-9654.

Bi, E. F. and J. Lutkenhaus (1991). "FtsZ ring structure associated with division in *Escherichia coli*." Nature 354(6349): 161-164.

Bockarie, M. J. and R. M. Deb (2010). "Elimination of lymphatic filariasis: do we have the drugs to complete the job?" Curr Opin Infect Dis 23(6): 617-620.

Brötz-Oesterhelt, H., D. Beyer, H. P. Kroll, R. Endermann, C. Ladel, W. Schroeder, B. Hinzen, S. Raddatz, H. Paulsen, K. Henninger, J. E. Bandow, H. G. Sahl and H. Labischinski (2005). "Dysregulation of bacterial proteolytic machinery by a new class of antibiotics." Nat Med 11(10): 1082-1087.

Brown, W. J. and D. D. Rockey (2000). "Identification of an antigen localized to an apparent septum within dividing *chlamydiae*." Infect Immun 68(2): 708-715.

Buist, G., A. Steen, J. Kok and O. P. Kuipers (2008). "LysM, a widely distributed protein motif for binding to (peptido)glycans." Mol Microbiol 68(4): 838-847.

Camberg, J. L., J. R. Hoskins and S. Wickner (2011). "The interplay of ClpXP with the cell division machinery in *Escherichia coli*." J Bacteriol 193(8): 1911-1918.

Carabeo, R. A., D. J. Mead and T. Hackstadt (2003). "Golgi-dependent transport of cholesterol to the *Chlamydia trachomatis* inclusion." Proc Natl Acad Sci U S A 100(11): 6771-6776.

Casiraghi, M., E. Ferri and C. Bandi (2007). *Wolbachia*: Evolutionary Significance in Nematodes. *Wolbachia*: A Bug's Life in another Bug. A. Hoerauf and R. U. Rao. Basel, Karger. 5**:** 15-30.

Cho, K. O., G. W. Kim and O. K. Lee (2011). "*Wolbachia* bacteria reside in host Golgi-related vesicles whose position is regulated by polarity proteins." PLoS One 6(7): e22703.

Clausen, T., R. Huber, A. Messerschmidt, H. D. Pohlenz and B. Laber (1997). "Slow-binding inhibition of *Escherichia coli* cystathionine beta-lyase by L-aminoethoxyvinylglycine: a kinetic and X-ray study." Biochemistry 36(41): 12633-12643.

Darby, A. C., S. D. Armstrong, G. S. Bah, G. Kaur, M. A. Hughes, S. M. Kay, P. Koldkjaer, A. D. Radford, M. L. Blaxter, V. N. Tanya, A. J. Trees, R. Cordaux, J. M. Wastling and B. L. Makepeace (2012). "Analysis of gene expression from the *Wolbachia* genome of a filarial nematode supports both metabolic and defensive roles within the symbiosis." Genome Res.

Datta, P., A. Dasgupta, A. K. Singh, P. Mukherjee, M. Kundu and J. Basu (2006). "Interaction between FtsW and penicillin-binding protein 3 (PBP3) directs PBP3 to mid-cell, controls cell septation and mediates the formation of a trimeric complex involving FtsZ, FtsW and PBP3 in *Mycobacteria*." Mol Microbiol 62(6): 1655-1673.

de Pedro, M. A., W. D. Donachie, J. V. Holtje and H. Schwarz (2001). "Constitutive septal murein synthesis in *Escherichia coli* with impaired activity of the morphogenetic proteins RodA and penicillin-binding protein 2." J Bacteriol 183(14): 4115-4126.

den Blaauwen, T., M. A. de Pedro, M. Nguyen-Disteche and J. A. Ayala (2008). "Morphogenesis of rod-shaped sacculi." FEMS Microbiol Rev 32(2): 321-344.

Dunning Hotopp, J. C., M. Lin, R. Madupu, J. Crabtree, S. V. Angiuoli, J. A. Eisen, R. Seshadri, Q. Ren, M. Wu, T. R. Utterback, S. Smith, M. Lewis, H. Khouri, C. Zhang, H. Niu, Q. Lin, N. Ohashi, N. Zhi, W. Nelson, L. M. Brinkac, R. J. Dodson, M. J. Rosovitz, J. Sundaram, S. C. Daugherty, T. Davidsen, A. S. Durkin, M. Gwinn, D. H. Haft, J. D. Selengut, S. A. Sullivan, N. Zafar, L. Zhou, F. Benahmed, H. Forberger, R. Halpin, S. Mulligan, J. Robinson, O. White, Y.

Rikihisa and H. Tettelin (2006). "Comparative genomics of emerging human ehrlichiosis agents." PLoS Genet 2(2): e21.

Fenn, K. and M. Blaxter (2006a). "Wolbachia genomes: revealing the biology of parasitism and mutualism." Trends Parasitol 22(2): 60-65.

Fenn, K., C. Conlon, M. Jones, M. A. Quail, N. E. Holroyd, J. Parkhill and M. Blaxter (2006b). "Phylogenetic relationships of the *Wolbachia* of nematodes and arthropods." PLoS Pathog 2(10): e94.

Fenollar, F., B. La Scola, H. Inokuma, J. S. Dumler, M. J. Taylor and D. Raoult (2003). "Culture and phenotypic characterization of a *Wolbachia pipientis* isolate." J Clin Microbiol 41(12): 5434-5441.

Foster, J., M. Ganatra, I. Kamal, J. Ware, K. Makarova, N. Ivanova, A. Bhattacharyya, V. Kapatral, S. Kumar, J. Posfai, T. Vincze, J. Ingram, L. Moran, A. Lapidus, M. Omelchenko, N. Kyrpides, E. Ghedin, S. Wang, E. Goltsman, V. Joukov, O. Ostrovskaya, K. Tsukerman, M. Mazur, D. Comb, E. Koonin and B. Slatko (2005). "The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode." PLoS Biol 3(4): e121.

Foster, J., S. Raverdy, M. B. Ganatra, P. A. Colussi, C. H. Taron and C. K. Carlow (2009). "The *Wolbachia* endosymbiont of *Brugia malayi* has an active phosphoglycerate mutase: a candidate target for anti-filarial therapies." Parasitol Res 104(5): 1047-1052.

Fraipont, C., S. Alexeeva, B. Wolf, R. van der Ploeg, M. Schloesser, T. den Blaauwen and M. Nguyen-Disteche (2010). "The integral membrane FtsW protein and peptidoglycan synthase PBP3 form a subcomplex in *Escherichia coli*." Microbiology 157(Pt 1): 251-259.

Francois, J. A. and T. J. Kappock (2007). "Alanine racemase from the acidophile *Acetobacter aceti*." Protein Expr Purif 51(1): 39-48.

Gaballah, A., A. Kloeckner, C. Otten, H. G. Sahl and B. Henrichfreise (2011). "Functional analysis of the cytoskeleton protein MreB from *Chlamydophila pneumoniae*." PLoS One 6(10): e25129.

Gerding, M. A., Y. Ogata, N. D. Pecora, H. Niki and P. A. de Boer (2007). "The trans-envelope Tol-Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in *E. coli*." Mol Microbiol 63(4): 1008-1025.

Godlewska, R., K. Wisniewska, Z. Pietras and E. K. Jagusztyn-Krynicka (2009). "Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria: function, structure, role in pathogenesis and potential application in immunoprophylaxis." FEMS Microbiol Lett 298(1): 1-11.

Goehring, N. W., F. Gueiros-Filho and J. Beckwith (2005). "Premature targeting of a cell division protein to midcell allows dissection of divisome assembly in *Escherichia coli*." Genes Dev 19(1): 127-137.

Gorbatyuk, B. and G. T. Marczynski (2005). "Regulated degradation of chromosome replication proteins DnaA and CtrA in *Caulobacter crescentus*." Mol Microbiol 55(4): 1233-1245.

Griswold, W. R. and M. D. Toney (2011). "Role of the pyridine nitrogen in pyridoxal 5'-phosphate catalysis: activity of three classes of PLP enzymes reconstituted with deazapyridoxal 5'-phosphate." J Am Chem Soc 133(37): 14823-14830.

Hasper, H. E., N. E. Kramer, J. L. Smith, J. D. Hillman, C. Zachariah, O. P. Kuipers, B. de Kruijff and E. Breukink (2006). "An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II." Science 313(5793): 1636-1637.

Heinzen, R. A., T. Hackstadt and J. E. Samuel (1999). "Developmental biology of *Coxiella burnetti*." Trends Microbiol 7(4): 149-154.

Henrichfreise, B., A. Schiefer, T. Schneider, E. Nzukou, C. Poellinger, T. J. Hoffmann, K. L. Johnston, K. Moelleken, I. Wiedemann, K. Pfarr, A. Hoerauf and H. G. Sahl (2009). "Functional conservation of the lipid II biosynthesis pathway in the cell wall-less bacteria *Chlamydia* and *Wolbachia*: why is lipid II needed?" Mol Microbiol 73(5): 913-923.

Hoerauf, A., S. Mand, O. Adjei, B. Fleischer and D. W. Buttner (2001). "Depletion of *Wolbachia* endobacteria in *Onchocerca volvulus* by doxycycline and microfilaridermia after ivermectin treatment." Lancet 357(9266): 1415-1416.

Hoerauf, A., K. Pfarr, S. Mand, A. Y. Debrah and S. Specht (2011). "Filariasis in Africa--treatment challenges and prospects." Clin Microbiol Infect 17(7): 977-985.

Hoerauf A, P. K. (2007). *Wolbachia* Endosymbionts: An Achilles' Heel of Filarial Nematodes. Wolbachia: A Bug's Life in another Bug. R. R. Hoerauf A. Basel, Karger. 5**:** 31-51.

Holtje, J. V. (1998). "Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*." Microbiol Mol Biol Rev 62(1): 181-203.

Hussain, M., F. D. Frentiu, L. A. Moreira, S. L. O'Neill and S. Asgari (2011). "*Wolbachia* uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector *Aedes aegypti*." Proc Natl Acad Sci U S A 108(22): 9250-9255.

Hyde, A. J., J. Parisot, A. McNichol and B. B. Bonev (2006). "Nisin-induced changes in *Bacillus* morphology suggest a paradigm of antibiotic action." Proc Natl Acad Sci U S A 103(52): 19896-19901.

Igarashi, A. (1978). "Isolation of a Singh's *Aedes albopictus* cell clone sensitive to *Dengue* and *Chikungunya* viruses." J Gen Virol 40(3): 531-544.

Joseleau-Petit, D., J. C. Liebart, J. A. Ayala and R. D'Ari (2007). "Unstable *Escherichia coli* L forms revisited: growth requires peptidoglycan synthesis." J Bacteriol 189(18): 6512-6520.

Jülicher, K. (2012). Untersuchung der potentiellen L-Aminosäure Racemasen MetC und GlyA in *Wolbachien*, Endobakterien aus Filarien Nematoden. Institute for Medical Microbiology, Immunology and Parasitology. Bonn, University Hospital Bonn. Bachelor thesis.

Kamgno, J., S. D. Pion, C. D. Mackenzie, B. Thylefors and M. Boussinesq (2009). "*Loa loa* microfilarial periodicity in ivermectin-treated patients: comparison between those developing and those free of serious adverse events." Am J Trop Med Hyg 81(6): 1056-1061.

Kang, L., A. C. Shaw, D. Xu, W. Xia, J. Zhang, J. Deng, H. F. Woldike, Y. Liu and J. Su (2011). "Upregulation of MetC is essential for D-alanine-independent growth of an alr/dadX-deficient *Escherichia coli* strain." J Bacteriol 193(5): 1098-1106.

Katabarwa, M. N., A. Eyamba, P. Nwane, P. Enyong, S. Yaya, J. Baldiagai, T. K. Madi, A. Yougouda, G. O. Andze and F. O. Richards (2011). "Seventeen years of annual distribution of ivermectin has not interrupted onchocerciasis transmission in North Region, Cameroon." Am J Trop Med Hyg 85(6): 1041-1049.

Katayama, N., H. Takano, M. Sugiyama, S. Takio, A. Sakai, K. Tanaka, H. Kuroiwa and K. Ono (2003). "Effects of antibiotics that inhibit the bacterial peptidoglycan synthesis pathway on moss chloroplast division." Plant Cell Physiol 44(7): 776-781.

Kersh, G. J., L. D. Oliver, J. S. Self, K. A. Fitzpatrick and R. F. Massung (2011). "Virulence of pathogenic *Coxiella burnetii* strains after growth in the absence of host cells." Vector Borne Zoonotic Dis 11(11): 1433-1438.

Kirkpatrick, C. L. and P. H. Viollier (2011). "New(s) to the (Z-)ring." Curr Opin Microbiol.

Kramer, M. J. and F. B. Gordon (1971). "Ultrastructural analysis of the effects of penicillin and chlortetracycline on the development of a genital tract *Chlamydia*." Infect Immun 3(2): 333-341.

Kuno, G. (1983). "Cultivation of mosquito cell lines in serum-free media and their effects on dengue virus replication." In Vitro 19(9): 707-713.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature 227(5259): 680-685.

Landmann, F., O. Bain, C. Martin, S. Uni, M. J. Taylor and W. Sullivan (2012). "Both asymmetric mitotic segregation and cell-to-cell invation are required for stable germline transmission of *Wolbachia* in filarial nematodes." Biology Open: 1-12.

Lee, B. G., E. Y. Park, K. E. Lee, H. Jeon, K. H. Sung, H. Paulsen, H. Rubsamen-Schaeff, H. Brotz-Oesterhelt and H. K. Song (2010). "Structures of ClpP in complex with acyldepsipeptide antibiotics reveal its activation mechanism." Nat Struct Mol Biol 17(4): 471-478.

Li, Z., A. L. Garner, C. Gloeckner, K. D. Janda and C. K. Carlow (2011). "Targeting the *Wolbachia* cell division protein FtsZ as a new approach for antifilarial therapy." PLoS Negl Trop Dis 5(11): e1411.

Lin, M. and Y. Rikihisa (2003). "*Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack genes for lipid A biosynthesis and incorporate cholesterol for their survival." Infect Immun 71(9): 5324-5331.

Lodish, H., A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore and J. Darnell (2000). 5.2 Purification of Cells and Their Parts. Molecular Cell Biology (4th edition). W. H. Freeman. New York, Palgrave Macmillan.

Luukkonen, A., M. Brummer-Korvenkontio and O. Renkonen (1973). "Lipids of cultured mosquito cells (*Aedes albopictus*). Comparison with cultured mammalian fibroblasts (BHK 21 cells)." Biochim Biophys Acta 326(2): 256-261.

Machida, M., K. Takechi, H. Sato, S. J. Chung, H. Kuroiwa, S. Takio, M. Seki, K. Shinozaki, T. Fujita, M. Hasebe and H. Takano (2006). "Genes for the peptidoglycan synthesis pathway are essential for chloroplast division in moss." Proc Natl Acad Sci U S A 103(17): 6753-6758.

Mackenzie, C. D., M. M. Homeida, A. D. Hopkins and J. C. Lawrence (2011). "Elimination of onchocerciasis from Africa: possible?" Trends Parasitol 28(1): 16-22.

Manohar, R., A. G. Rao and N. A. Rao (1984). "Kinetic mechanism of the interaction of D-cycloserine with serine hydroxymethyltransferase." Biochemistry 23(18): 4116-4122.

Margolin, W. (2005). "FtsZ and the division of prokaryotic cells and organelles." Nat Rev Mol Cell Biol 6(11): 862-871.

Matsumoto, A. and G. P. Manire (1970). "Electron microscopic observations on the effects of penicillin on the morphology of *Chlamydia* psittaci." J Bacteriol 101(1): 278-285.

Mavingui, P., C. Valiente Moro, V. Tran-Van, F. Wisniewski-Dye, V. Raquin, G. Minard, F. H. Tran, D. Voronin, Z. Rouy, P. Bustos, L. Lozano, V. Barbe and V. Gonzalez (2012). "Whole-genome sequence of *Wolbachia* strain *w*AlbB, an endosymbiont of tiger mosquito vector *Aedes albopictus*." J Bacteriol 194(7): 1840.

McGraw, E. A., D. J. Merritt, J. N. Droller and S. L. O'Neill (2002). "*Wolbachia* density and virulence attenuation after transfer into a novel host." Proc Natl Acad Sci U S A 99(5): 2918-2923.

McMeniman, C. J., A. M. Lane, A. W. Fong, D. A. Voronin, I. Iturbe-Ormaetxe, R. Yamada, E. A. McGraw and S. L. O'Neill (2008). "Host adaptation of a *Wolbachia* strain after long-term serial passage in mosquito cell lines." Appl Environ Microbiol 74(22): 6963-6969.

McNulty, S. N., J. M. Foster, M. Mitreva, J. C. Dunning Hotopp, J. Martin, K. Fischer, B. Wu, P. J. Davis, S. Kumar, N. W. Brattig, B. E. Slatko, G. J. Weil and P. U. Fischer (2010). "Endosymbiont DNA in endobacteria-free filarial nematodes indicates ancient horizontal genetic transfer." PLoS One 5(6): e11029.

Mengome, L. E., J. P. Akue, A. Souza, G. R. Feuya Tchoua and E. Nsi Emvo (2010). "*In vitro* activities of plant extracts on human *Loa loa* isolates and cytotoxicity for eukaryotic cells." Parasitol Res 107(3): 643-650.

Milner, Y. and H. G. Wood (1976). "Steady state and exchange kinetics of pyruvate, phosphate dikinase from *Propionibacterium shermanii*." J Biol Chem 251(24): 7920-7928.

Min, K. T. and S. Benzer (1997). "*Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death." Proc Natl Acad Sci U S A 94(20): 10792-10796.

Mohammadi, T., V. van Dam, R. Sijbrandi, T. Vernet, A. Zapun, A. Bouhss, M. Diepeveen-de Bruin, M. Nguyen-Disteche, B. de Kruijff and E. Breukink (2011). "Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane." EMBO J 30(8): 1425-1432.

Moulder, J. W. (1993). "Why is *Chlamydia* sensitive to penicillin in the absence of peptidoglycan?" Infect Agents Dis 2(2): 87-99.

Omsland, A., D. C. Cockrell, D. Howe, E. R. Fischer, K. Virtaneva, D. E. Sturdevant, S. F. Porcella and R. A. Heinzen (2009). "Host cell-free growth of the Q fever bacterium *Coxiella burnetii*." Proc Natl Acad Sci U S A 106(11): 4430-4434.

Park, I. S., J. H. Kim and B. G. Kim (2005). "The effects of ftsZ mutation on the production of recombinant protein in *Bacillus subtilis*." Appl Microbiol Biotechnol 69(1): 57-64.
Park, J. T. and T. Uehara (2008). "How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan)." Microbiol Mol Biol Rev 72(2): 211-227.
Parsons, L. M., F. Lin and J. Orban (2006). "Peptidoglycan recognition by Pal, an outer membrane lipoprotein." Biochemistry 45(7): 2122-2128.
Pfarr, K., A. Y. Debrah, S. Specht and A. Hoerauf (2009). "Filariasis and lymphoedema." Parasite Immunol 31(11): 664-672.
Pfarr, K. and A. Hoerauf (2005). "The annotated genome of *Wolbachia* from the filarial nematode *Brugia malayi*: what it means for progress in antifilarial medicine." PLoS Med 2(4): e110.
Popov, V. L., V. C. Han, S. M. Chen, J. S. Dumler, H. M. Feng, T. G. Andreadis, R. B. Tesh and D. H. Walker (1998). "Ultrastructural differentiation of the genogroups in the genus *Ehrlichia*." J Med Microbiol 47(3): 235-251.
Raju, R. M., M. Unnikrishnan, D. H. Rubin, V. Krishnamoorthy, O. Kandror, T. N. Akopian, A. L. Goldberg and E. J. Rubin (2012). "*Mycobacterium tuberculosis* ClpP1 and ClpP2 function together in protein degradation and are required for viability *in vitro* and during infection." PLoS Pathog 8(2): e1002511.
Rasgon, J. L., C. E. Gamston and X. Ren (2006). "Survival of *Wolbachia pipientis* in cell-free medium." Appl Environ Microbiol 72(11): 6934-6937.
Rolls, M. M., M. T. Marquardt, M. Kielian and C. E. Machamer (1997). "Cholesterol-independent targeting of Golgi membrane proteins in insect cells." Mol Biol Cell 8(11): 2111-2118.
Ruang-areerate, T., P. Kittayapong, E. A. McGraw, V. Baimai and S. L. O'Neill (2004). "*Wolbachia* replication and host cell division in *Aedes albopictus*." Curr Microbiol 49(1): 10-12.
Sass, P., M. Josten, K. Famulla, G. Schiffer, H. G. Sahl, L. Hamoen and H. Brötz-Oesterhelt (2011). "Antibiotic acyldepsipeptides activate ClpP peptidase to degrade the cell division protein FtsZ." Proc Natl Acad Sci U S A 108(42): 17474-17479.
Sauerzweig, S., K. Baldauf, H. Braun and K. G. Reymann (2009). "Time-dependent segmentation of BrdU-signal leads to late detection problems in studies using BrdU as cell label or proliferation marker." J Neurosci Methods 177(1): 149-159.
Scheffers, D. J. and M. G. Pinho (2005). "Bacterial cell wall synthesis: new insights from localization studies." Microbiol Mol Biol Rev 69(4): 585-607.
Schiefer, A., J. Vollmer, C. Lämmer, S. Specht, C. Lentz, H. Ruebsamen-Schaeff, H. Brötz-Oesterhelt, A. Hoerauf and K. Pfarr (2012, submitted). "The ClpP Peptidase of *Wolbachia* Endobacteria is a novel Target for Drug Development against Filarial Infections." submitted.
Serbus, L. R. and W. Sullivan (2007). "A cellular basis for *Wolbachia* recruitment to the host germline." PLoS Pathog 3(12): e190.
Singh, K. R. P. (1967). "Cell Cultures Derived From Larvae Of *Aedes Albopictus* (SKUSE) and *Aedes aegypti* " Curr Sci 36: 506-507.
Skilton, R. J., L. T. Cutcliffen, D. Barlow, Y. Wang, O. Salim, P. R. Lambden and I. N. Clarke (2009). "Penicillin induced persistence in *Chlamydia trachomatis*: high quality time lapse video analysis of the developmental cycle." PLoS One 4(11): e7723.
Slatko, B. E., M. J. Taylor and J. M. Foster (2010). "The *Wolbachia* endosymbiont as an anti-filarial nematode target." Symbiosis 51(1): 55-65.
Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. Zhao, E. V. Koonin and R. W. Davis (1998). "Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*." Science 282(5389): 754-759.
Strübing, U., R. Lucius, A. Hoerauf and K. M. Pfarr (2010). "Mitochondrial genes for heme-dependent respiratory chain complexes are up-regulated after depletion of *Wolbachia* from filarial nematodes." Int J Parasitol 40(10): 1193-1202.

Suginaka, H., P. M. Blumberg and J. L. Strominger (1972). "Multiple penicillin-binding components in *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli*." J Biol Chem 247(17): 5279-5288.

Takahata, S., T. Ida, T. Hiraishi, S. Sakakibara, K. Maebashi, S. Terada, T. Muratani, T. Matsumoto, C. Nakahama and K. Tomono (2010). "Molecular mechanisms of fosfomycin resistance in clinical isolates of *Escherichia coli*." Int J Antimicrob Agents 35(4): 333-337.

Tamarozzi, F., A. Halliday, K. Gentil, A. Hoerauf, E. Pearlman and M. J. Taylor (2011). "Onchocerciasis: the role of *Wolbachia* bacterial endosymbionts in parasite biology, disease pathogenesis, and treatment." Clin Microbiol Rev 24(3): 459-468.

Taylor, M. J. and A. Hoerauf (1999). "*Wolbachia* bacteria of filarial nematodes." Parasitol Today 15(11): 437-442.

Taylor, M. J., A. Hoerauf and M. Bockarie (2010). "Lymphatic filariasis and onchocerciasis." Lancet 376(9747): 1175-1185.

Torruella, G., H. Suga, M. Riutort, J. Pereto and I. Ruiz-Trillo (2009). "The evolutionary history of lysine biosynthesis pathways within eukaryotes." J Mol Evol 69(3): 240-248.

Turner, J. D., R. S. Langley, K. L. Johnston, G. Egerton, S. Wanji and M. J. Taylor (2006). "*Wolbachia* endosymbiotic bacteria of *Brugia malayi* mediate macrophage tolerance to TLR- and CD40-specific stimuli in a MyD88/TLR2-dependent manner." J Immunol 177(2): 1240-1249.

Turner, J. D., R. S. Langley, K. L. Johnston, K. Gentil, L. Ford, B. Wu, M. Graham, F. Sharpley, B. Slatko, E. Pearlman and M. J. Taylor (2009). "*Wolbachia* lipoprotein stimulates innate and adaptive immunity through Toll-like receptors 2 and 6 to induce disease manifestations of filariasis." J Biol Chem 284(33): 22364-22378.

Typas, A., M. Banzhaf, C. A. Gross and W. Vollmer (2011). "From the regulation of peptidoglycan synthesis to bacterial growth and morphology." Nat Rev Microbiol 10(2): 123-136.

Typas, A., M. Banzhaf, B. van den Berg van Saparoea, J. Verheul, J. Biboy, R. J. Nichols, M. Zietek, K. Beilharz, K. Kannenberg, M. von Rechenberg, E. Breukink, T. den Blaauwen, C. A. Gross and W. Vollmer (2010). "Regulation of peptidoglycan synthesis by outer-membrane proteins." Cell 143(7): 1097-1109.

Vinella, D., D. Joseleau-Petit, D. Thevenet, P. Bouloc and R. D'Ari (1993). "Penicillin-binding protein 2 inactivation in *Escherichia coli* results in cell division inhibition, which is relieved by FtsZ overexpression." J Bacteriol 175(20): 6704-6710.

Vollmer, J., A. Schiefer, T. Schneider, K. Jülicher, K. L. Johnston, M. J. Taylor, H.-G. Sahl, A. Hoerauf and K. Pfarr (2013). "Cell Wall-Less *Wolbachia*, Endobacteria of Arthropods and Filarial Nematodes, Require Lipid II Biosynthesis for Cell Division." accepted in Int J Med Microbiol (DOI: 10.1016/j.ijmm.2013.01.002).

Vollmer, W. and U. Bertsche (2008a). "Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli*." Biochim Biophys Acta 1778(9): 1714-1734.

Vollmer, W., D. Blanot and M. A. de Pedro (2008b). "Peptidoglycan structure and architecture." FEMS Microbiol Rev 32(2): 149-167.

Wang, X. and J. Lutkenhaus (1993). "The FtsZ protein of *Bacillus subtilis* is localized at the division site and has GTPase activity that is dependent upon FtsZ concentration." Mol Microbiol 9(3): 435-442.

Wenk, P. and A. Renz (2003). 2.3 Filarien mit zirkulierenden Mikrofilarien. Parasitologie. Biologie der Humanparasiten Stuttgart, Thieme**:** 51-65.

White, C. L., A. Kitich and J. W. Gober (2010). "Positioning cell wall synthetic complexes by the bacterial morphogenetic proteins MreB and MreD." Mol Microbiol 76(3): 616-633.

WHO (2010). "Global Programme to Eliminate Lymphatic Filariasis." Progress report 2000-2009 and strategic plan 2010-2020 of the global programme to eliminate lymphatic filariasis: halfway towards eliminating lymphatic filariasis.

WHO (2011). ""African Programme for Onchocerciasis Control: meeting of national task forces, September 2011."" Weekly epidemiological record(86(48)): 541-549.

Wright, J. D. and A. R. Barr (1980). "The ultrastructure and symbiotic relationships of *Wolbachia* of mosquitoes of the *Aedes scutellaris* group." J Ultrastruct Res 72(1): 52-64.

Wright, J. D., F. S. Sjostrand, J. K. Portaro and A. R. Barr (1978). "The ultrastructure of the rickettsia-like microorganism *Wolbachia pipientis* and associated virus-like bodies in the mosquito *Culex pipiens*." J Ultrastruct Res 63(1): 79-85.

Wu, B., J. Novelli, J. Foster, R. Vaisvila, L. Conway, J. Ingram, M. Ganatra, A. U. Rao, I. Hamza and B. Slatko (2009). "The heme biosynthetic pathway of the obligate *Wolbachia* endosymbiont of *Brugia malayi* as a potential anti-filarial drug target." PLoS Negl Trop Dis 3(7): e475.

Wu, M., L. V. Sun, J. Vamathevan, M. Riegler, R. Deboy, J. C. Brownlie, E. A. McGraw, W. Martin, C. Esser, N. Ahmadinejad, C. Wiegand, R. Madupu, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, A. S. Durkin, J. F. Kolonay, W. C. Nelson, Y. Mohamoud, P. Lee, K. Berry, M. B. Young, T. Utterback, J. Weidman, W. C. Nierman, I. T. Paulsen, K. E. Nelson, H. Tettelin, S. L. O'Neill and J. A. Eisen (2004). "Phylogenomics of the reproductive parasite *Wolbachia pipientis wMel*: a streamlined genome overrun by mobile genetic elements." PLoS Biol 2(3): E69.

Yamada, S., Y. Hyo, S. Ohmori and M. Ohuchi (2007). "Role of ciprofloxacin in its synergistic effect with fosfomycin on drug-resistant strains of *Pseudomonas aeruginosa*." Chemotherapy 53(3): 202-209.

Yeh, Y. C., L. R. Comolli, K. H. Downing, L. Shapiro and H. H. McAdams (2010). "The *caulobacter* Tol-Pal complex is essential for outer membrane integrity and the positioning of a polar localization factor." J Bacteriol 192(19): 4847-4858.

Zug, R. and P. Hammerstein (2012). "Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected." PLoS One 7(6): e38544.

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