Antibiotic-induced cellular effects in *Wolbachia*: Insights from cell wall biosynthesis inhibitors and corallopyronin A

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“All zoology is really ecology.

We cannot fully understand the lives of animals

without understanding our microbes and our symbioses with them.”

(Ed Yong)

Summary

Anti-*Wolbachia* therapy is a powerful strategy to treat the human infections lymphatic filariasis and onchocerciasis, neglected tropical diseases caused by filarial worms that are major drivers of morbidity and poverty. *Wolbachia*, obligate endobacteria of these worms, are essential for embryogenesis, larval development, and adult survival. Despite their highly reduced genome and lack of a canonical cell wall, *Wolbachia* retain key enzymes for lipid II synthesis.

This thesis investigates the cellular effects of cell wall biosynthesis inhibitors and corallopyronin A (CorA) in *Wolbachia*. The first publication "*In vitro* extracellular replication of *Wolbachia* endobacteria" establishes a host cell-free *Wolbachia* culture. Host cell lysate enabled extracellular growth of *Wolbachia* for 12 days. Here, fosfomycin, which inhibits the first dedicated enzyme in lipid II biosynthesis (MurA), induced enlarged extracellular *Wolbachia*, as seen for intracellular *Wolbachia*, whereas bacitracin, vancomycin, and ampicillin had no observable effects.

The second publication "MraY inhibitor muraymycin D2 and derivatives induce enlarged cells in obligate intracellular *Chlamydia* and *Wolbachia* and break persistence in *Chlamydia*" explores the cellular effects of muraymycins, inhibitors of MraY, the enzyme catalyzing the first membrane-bound step of lipid II biosynthesis. Two muraymycin derivatives inhibited growth of *Wolbachia* and induced an enlarged phenotype, similar to fosfomycin, identifying MraY as a new wolbachial target for drug development.

The third publication "No resistance development against corallopyronin A in *Wolbachia* in C6/36 cell culture" assesses the potential for resistance development against the RNA polymerase inhibitor CorA, which is in preclinical development against human filarial infections. *Wolbachia* were exposed to CorA for 245 days. No reduction in CorA efficacy was observed, suggesting a low frequency of mutation to resistance and supporting its further development.

The manuscript "Improved RNA preparation for RNA-seq of the intracellular bacterium *Wolbachia w*AlbB" identifies conditions that yielded high-quality RNA for RNA-seq, with custom-designed riboPOOLs effectively depleting rRNA. Combined with host cell mRNA

depletion using Dynabeads, 30.2% of reads mapped to *Wolbachia*, a 300-fold increase compared to our first RNA-seq experiment.

By investigating the cellular effects of cell wall biosynthesis inhibitors and CorA in *Wolbachia*, this thesis provides insights into *Wolbachia* biology. Key findings include the establishment of a host cell-free *Wolbachia* culture, muraymycin-induced enlargement of *Wolbachia*, further supporting that lipid II is essential for their cell division, and the lack of resistance development against CorA in prolonged cell culture. Additionally, an optimized workflow for RNA-seq of *Wolbachia* will enable detailed transcriptomic studies, including the investigation of cellular effects of antibiotics.

Zusammenfassung

Die Anti-*Wolbachia*-Therapie hat sich als effektive Strategie zur Behandlung von Infektionen des Menschen mit lymphatischer Filariose und Onchozerkose etabliert. Diese zählen zu den vernachlässigten Tropenkrankheiten, werden durch Filarienwürmer verursacht und stellen eine bedeutende Ursache für Morbidität und Armut dar. *Wolbachia* (im Deutschen auch: Wolbachien), obligate Endobakterien dieser Würmer, sind für die Embryogenese, die Larvenentwicklung und das Überleben der adulten Würmer unerlässlich. Obwohl ihr Genom stark reduziert ist und keine kanonische Zellwand vorliegt, verfügen Wolbachien über Schlüsselenzyme für die Lipid-II-Biosynthese.

In dieser Dissertation werden die zellulären Effekte von Zellwandbiosynthese-Inhibitoren und Corallopyronin A (CorA) in Wolbachien untersucht. Das erste Paper „*In vitro* extracellular replication of *Wolbachia* endobacteria“ beschreibt die Etablierung einer wirtszellfreien *Wolbachia*-Kultur. Mithilfe von Wirtszelllysat wurde extrazelluläres Wachstum von Wolbachien für bis zu 12 Tage ermöglicht. Fosfomycin, welches das erste dedizierte Enzym der Lipid-II-Biosynthese (MurA) hemmt, induzierte eine Vergrößerung der extrazellulären Wolbachien, wie sie zuvor für intrazelluläre beobachtet wurde, während Bacitracin, Vancomycin und Ampicillin keine beobachtbaren Effekte hatten.

Das zweite Paper „MraY inhibitor muraymycin D2 and derivatives induce enlarged cells in obligate intracellular *Chlamydia* and *Wolbachia* and break persistence in *Chlamydia*“ untersucht die zellulären Effekte von Muraymycinen, Inhibitoren von MraY, dem Enzym, das den ersten membrangebundenen Schritt der Lipid-II-Biosynthese katalysiert. Zwei Muraymycin-Derivate hemmten das Wachstum von Wolbachien in Zellkultur und induzierten einen vergrößerten Phänotyp, ähnlich wie Fosfomycin, wodurch MraY als neue Zielstruktur für die Entwicklung von Medikamenten gegen Wolbachien identifiziert wurde.

Das dritte Paper „No resistance development against corallopyronin A in *Wolbachia* in C6/36 cell culture“ bewertet das Potenzial zur Resistenzentwicklung gegenüber dem RNA-Polymerase-Inhibitor CorA, der sich in der präklinischen Entwicklung gegen humane Filarieninfektionen befindet. Wolbachien wurden 245 Tage lang mit CorA behandelt. Keine Abnahme der Wirksamkeit von CorA wurde beobachtet, was auf eine geringe Frequenz

resistenzvermittelnder Mutationen hinweist und die Weiterentwicklung des Wirkstoffs unterstützt.

Das Manuskript „Improved RNA preparation for RNA-seq of the intracellular bacterium *Wolbachia w*AlbB“ identifiziert Bedingungen, die qualitativ hochwertige RNA für RNA-Sequenzierung (RNA-Seq) liefern, wobei maßgeschneiderte riboPOOLs die rRNA effektiv depletierten. In Kombination mit der Depletion der Wirtszell-mRNA mittels Dynabeads konnten 30,2% *Wolbachia*-Reads erzielt werden, was einem 300-fachen Anstieg im Vergleich zu unserem ersten RNA-Seq-Experiment entspricht.

Mit der Untersuchung der zellulären Effekte von Zellwandbiosynthese-Inhibitoren und CorA in *Wolbachia* liefert diese Dissertation Einblicke in die Biologie von Wolbachien. Zu den wichtigsten Ergebnissen gehören die Etablierung einer wirtszellfreien *Wolbachia*-Kultur, die Muraymycin-induzierte Vergrößerung von Wolbachien, was die essenzielle Rolle von Lipid II in ihrer Zellteilung weiter untermauert, und das Ausbleiben einer Resistenzentwicklung gegenüber CorA in Langzeitkultur. Darüber hinaus ermöglicht ein optimierter Prozess für RNA-Seq von Wolbachien zukünftig detaillierte Transkriptomstudien, einschließlich der Untersuchung zellulärer Effekte von Antibiotika.

Table of Contents

List of Abbreviations

A. aegypti	*Aedes aegypti*
A. albopictus	*Aedes albopictus*
A. phagocytophilum	*Anaplasma phagocytophilum*
A. viteae	*Acanthocheilonema viteae*
AB	aberrant body
ALB	albendazole
AMR	antimicrobial resistance
aPBP	class A penicillin-binding protein
B. malayi	*Brugia malayi*
B. pahangi	*Brugia pahangi*
bPBP	class B penicillin-binding protein
C. burnetii	*Coxiella burnetii*
C. pneumoniae	*Chlamydia pneumoniae*
C. trachomatis	*Chlamydia trachomatis*
C_{55}-P	undecaprenyl phosphate
CorA	corallopyronin A
CRISPRi	CRISPR interference
DALY	disability-adjusted life year
Ddl	D-Ala:D-Ala ligase
DEC	diethylcarbamazine
D-Glu	D-glutamate
DOX	doxycycline
E. coli	*Escherichia coli*
EDA	ethynyl-D-alanine
FBS	fetal bovine serum
FOS	fosfomycin
Glc*N*Ac	*N*-acetylglucosamine
IVM	ivermectin
L. loa	*Loa loa*

L. sigmodontis	*Litomosoides sigmodontis*
L-Ala	L-alanine
LF	lymphatic filariasis
LPS	lipopolysaccharide
Mb	megabase
MDA	mass drug administration
m-DAP	*meso*-diaminopimelate
Mf	microfilariae
MIC	minimum inhibitory concentration
MS	mass spectrometry
Mur*N*Ac	*N*-acetylmuramic acid
N. gonorrhoeae	*Neisseria gonorrhoeae*
NOD	nucleotide-binding oligomerization domain-containing protein
NTD	neglected tropical disease
O. tsutsugamushi	*Orientia tsutsugamushi*
O. volvulus	*Onchocerca volvulus*
PBP	penicillin-binding protein
PC	preventive chemotherapy
PGN	peptidoglycan
RNA-seq	RNA sequencing
S. aureus	*Staphylococcus aureus*
SEDS	shape, elongation, division, and sporulation family
sp.	species
UDP	uridine diphosphate
UPLC	ultra-performance liquid chromatography
W. bancrofti	*Wuchereria bancrofti*
*w*AlbB	*Wolbachia* strain B of *Aedes albopictus*
*w*Bm	*Wolbachia* of *Brugia malayi*
WHO	World Health Organization
*w*Mel	*Wolbachia* of *Drosophila melanogaster*
*w*Str	*Wolbachia* of *Laodelphax striatellus*

List of Figures

List of Tables

1 Introduction

1.1 Neglected tropical diseases

Neglected tropical diseases (NTDs) encompass a wide range of conditions of parasitic, bacterial, viral, fungal and non-communicable origin that primarily affect populations in low-income and resource-limited regions of the world (Mackey et al., 2014; Hotez et al., 2020; WHO, 2020). The term “neglected” highlights the limited attention these diseases have historically received with regard to funding, research, and healthcare infrastructure compared to other global health priorities (WHO, 2004; Liese et al., 2010; WHO, 2010). It is estimated that more than one billion people are affected by NTDs, with a significant burden in developing countries in tropical and subtropical regions (WHO, 2024a). NTDs disproportionately affect “the poorest of the poor”, as they thrive in environments characterized by poor sanitation, limited access to clean water, and inadequate healthcare systems, often perpetuating cycles of poverty and marginalization (WHO, 2004, 2006; Houweling et al., 2016). Among the currently 21 NTDs recognized by the World Health Organization (WHO), filariasis is a significant contributor to the global disease burden (WHO, 2024a). The two primary diseases caused by filarial parasites, lymphatic filariasis and onchocerciasis, affect millions and require targeted control and treatment strategies (see 1.1.1 and 1.1.2).

Efforts to address NTDs have gained momentum over the past two decades, driven by initiatives of the WHO, United Nations, and various global partnerships (Mackey et al., 2014; Molyneux et al., 2017; Hudu et al., 2024). In 2007, a first meeting of global partners took place, which resulted in the *Global Plan to combat neglected tropical diseases 2008–2015* (WHO, 2007). In 2012, the WHO published its first road map for NTDs (2012–2020) to accelerate the control and elimination of NTDs (WHO, 2012), with private and public partners committing to work on WHO targets by signing the London Declaration on NTDs (Hotez, 2013; WHO, 2013). In 2015, the United Nations included NTDs as a Sustainable Development Goal, with the aim of ending the epidemics by 2030 (UN, 2015). The WHO road map for NTDs 2021–2030 sets goals and milestones to prevent, control, eliminate, or eradicate NTDs in alignment with the Sustainable Development Goals (WHO, 2020). As of

February 2025, 55 countries have eliminated at least one NTD – more than half of the elimination target of 100 countries set for 2030 (WHO, 2025a). However, 1.62 billion people still required interventions against NTDs in 2022 (WHO, 2024a). While this is a 26% decrease since 2010, the road map's global target is a 90% reduction by 2030 (WHO, 2020). Challenges include sustaining funding, overcoming drug resistance, and reaching remote or conflict-affected areas (WHO, 2020). NTDs on the verge of elimination remain at risk of resurgence due to conflicts, economic instability, poverty, migration, and climate change (WHO, 2020; Hudu et al., 2024).

1.1.1 Lymphatic filariasis and onchocerciasis

Lymphatic filariasis (LF), commonly known as elephantiasis, and onchocerciasis, also known as river blindness, are infectious diseases caused by filariae, thread-like parasitic nematodes. These diseases collectively affect millions of individuals, primarily in tropical and subtropical regions (see 1.1.2), and are responsible for substantial social, economic, and health burdens (Taylor et al., 2010; Mathew et al., 2020; WHO, 2020; Frallonardo et al., 2022). As of 2023, around 657 million people across 39 countries, mainly in Africa and Southeast Asia, were at risk of LF (WHO, 2024b). In parallel, 249.5 million in 28 countries were at risk of onchocerciasis (WHO, 2024c). It is important to recognize that these figures may overlap, as regions endemic for both diseases exist. Around 51.4 million people were living with LF (Local Burden of Disease 2019 Neglected Tropical Diseases Collaborators, 2020), with an estimated 16.7 million suffering from lymphedema and 19.4 million men suffering from hydrocele (Ramaiah and Ottesen, 2014). At the same time, around 21 million were living with onchocerciasis (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018), with over 99% of onchocerciasis cases in sub-Saharan Africa (WHO, 2024c). In 2017, the Global Burden of Disease Study estimated that among those living with onchocerciasis, 14.7 million had skin disease and 1.2 million experienced vision loss (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018).

Although LF and onchocerciasis are not directly fatal, they cause significant disability and social and economic challenges. Disability-adjusted life years (DALYs) regard the immense limitations to the quality of life for infected people and measure the impact of the disease

on the affected community. LF contributes 1.6 million DALYs (WHO, 2020) and onchocerciasis 1.3 million DALYs (GBD 2017 DALYs and HALE Collaborators, 2018). For comparison, the latter study reported 1.3 million DALYs for non-melanoma skin cancer, 1.1 million DALYs for multiple sclerosis, and 0.36 million DALYs for sexually transmitted chlamydial infections (GBD 2017 DALYs and HALE Collaborators, 2018).

LF is caused by three filarial species: *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, with approximately 90% of cases attributed to *W. bancrofti* (Michael et al., 1996; Taylor et al., 2010). Transmission occurs via mosquito vectors, belonging to the genera *Anopheles*, *Culex*, *Aedes*, and *Mansonia* (Hoerauf et al., 2011). Onchocerciasis, caused by *Onchocerca volvulus*, is transmitted by blackflies of the genus *Simulium*. These blackflies breed near fast-flowing rivers, which limits the distribution of onchocerciasis to areas within a few kilometers of these breeding sites, hence the name river blindness (Büttner, 1967; Renz et al., 1987; Noma et al., 2002).

Filariae share a common life cycle involving an arthropod intermediate and a vertebrate definitive host. For LF- and onchocerciasis-causing nematodes, humans are the definitive hosts. Their life cycle is illustrated in Fig. 1. During a blood meal, infective third-stage (L3) larvae from an infected arthropod are transmitted onto the skin of the human host. L3 larvae penetrate through the bite wound and migrate to the lymphatic system (LF) or the subcutaneous tissues (onchocerciasis). There, they molt twice as they develop into adult worms (Wenk and Renz, 2003). Adult female worms can survive for prolonged periods, estimated as up to ten years in LF and up to 15 years in onchocerciasis (Roberts et al., 1967; Omura and Crump, 2004; Paily et al., 2009; Davis et al., 2019). Sexual reproduction of the adult worms produces microfilariae (Mf), which migrate to peripheral blood (LF) or skin and lymphatics (onchocerciasis). Mf are then taken up by another vector during a subsequent blood meal. In the vector, Mf develop into L3 larvae, which migrate into the head of the vector, more specifically to the mouthpart (proboscis), and are transmitted to a human host via another bite (Fischer et al., 2023), initiating a new cycle. However, it typically requires multiple bites to establish an infection; therefore, short-term travelers have a low risk (Chandra, 2008; Kapoor et al., 2015; Rebollo and Bockarie, 2017).

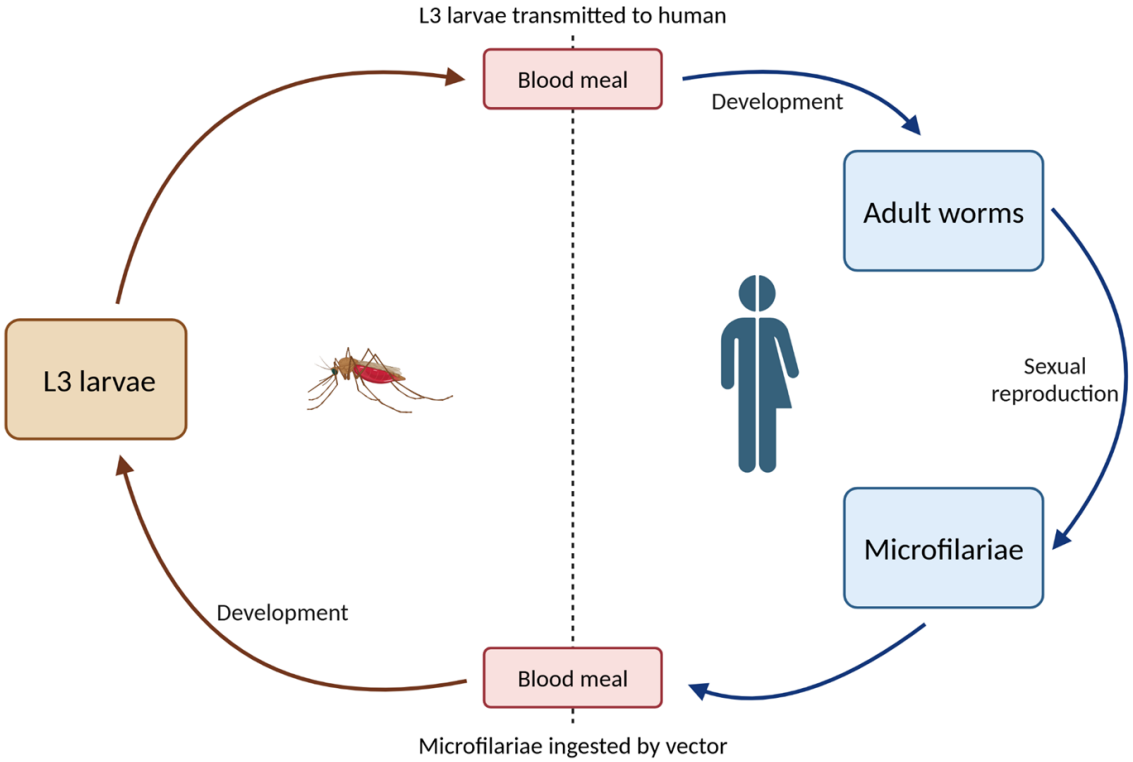

Figure 1. Life cycle of human filarial nematodes. Infective third-stage (L3) larvae are transmitted by an infected arthropod vector during a blood meal. L3 larvae then migrate to the lymphatic system (lymphatic filariasis) or the subcutaneous tissues (onchocerciasis) where they develop into adult worms. Adult filariae mate and release microfilariae that migrate to peripheral blood (lymphatic filariasis) or skin and lymphatics (onchocerciasis), from where they can be taken up by another vector during a blood meal. Inside the vector, microfilariae develop into L3 larvae after which they can infect another human during a blood meal, thus completing the life cycle. Adapted from Fischer et al. (2023) and created with BioRender.com.

The clinical symptoms of LF and onchocerciasis arise primarily from the immune response to dead and dying adult worms and Mf, respectively (Ottesen, 1995; Dreyer et al., 2000; Taylor et al., 2010). Although in LF, live adult worms in lymphatic vessels, particularly in the extremities and male genitalia, lead to dilation of these vessels, degenerate Mf trigger the release of pro-inflammatory cytokines and vascular endothelial growth factors that promote lymphangiogenesis (Pfarr et al., 2009; Taylor et al., 2010). The impaired function of these enlarged vessels can result in lymphedema (Fig. 2A) and hydrocele, and it predisposes the patients for secondary bacterial infections (Dreyer et al., 2000; Pfarr et al., 2009; Babu and Nutman, 2012). LF has been considered the world's leading cause of

physical disability and is associated with severe stigma and marginalization (Molyneux and Zagaria, 2002; Zeldenryk et al., 2011).

In onchocerciasis, adult worms reside in subcutaneous nodules (Fig. 2B). Female *O. volvulus* release around 1000 Mf per day, which migrate through skin and ocular tissues (Brattig, 2004; Makepeace et al., 2015; Fischer et al., 2023). The death of Mf induces immune responses that can cause a variety of pathologies including blindness (river blindness), skin rashes, lesions, intense itching, and skin depigmentation (sowda) (Taylor et al., 2010). These inflammatory immune responses are influenced by the release of the endosymbiotic *Wolbachia* bacteria (Saint André et al., 2002; Tamarozzi et al., 2011; Bouchery et al., 2013) (see 1.2.2). River blindness is the second leading infectious cause of blindness globally (Boatin and Richards, 2006; WHO, 2024c). Ongoing research describes an association between *O. volvulus* infection and epilepsy, including nodding syndrome, a poorly understood seizure disorder mostly affecting children in sub-Saharan Africa, though a causal relationship remains unproven (Foltz et al., 2013; Abd-Elfarag et al., 2021; Arndts et al., 2023; Hadermann et al., 2023).

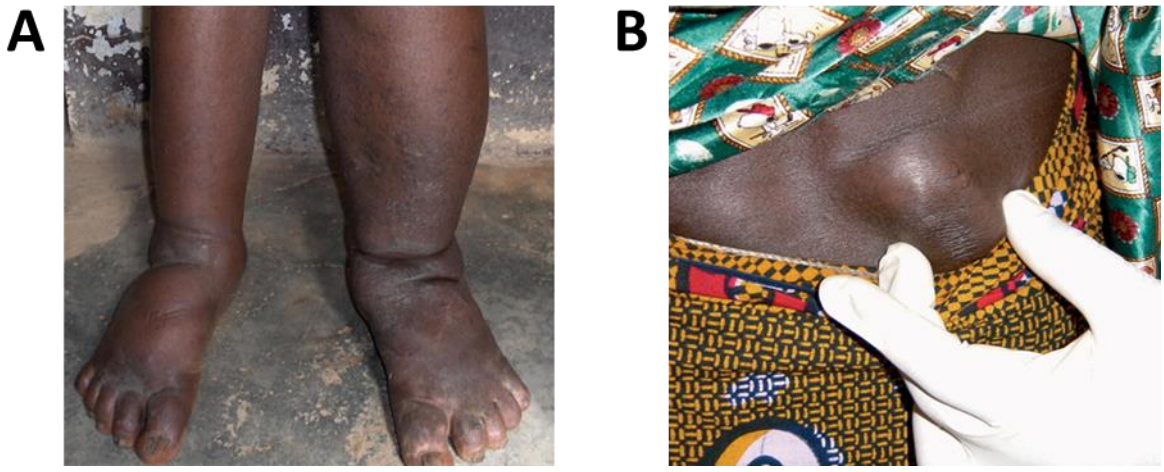

Figure 2. Clinical signs of lymphatic filariasis and onchocerciasis. (**A**) In lymphatic filariasis, death of adult worms (*Wuchereria bancrofti*, *Brugia malayi* or *Brugia timori*) inside lymphatic vessels can lead to lymphedema. The image was adapted from Hübner et al. (2022a). (**B**) In onchocerciasis, worms of the species *Onchocerca volvulus* reside in subcutaneous nodules. The image was adapted from Taylor et al. (2010).

1.1.2 Control and treatment of filarial diseases

In 1997, WHO and its member states made a commitment to eliminate LF as public health problem by 2020. Consequently, the *Global Programme to Eliminate Lymphatic Filariasis* (GPELF) was launched in 2000 (Ramaiah and Ottesen, 2014). The goal has not been met; the current road map of the WHO sets 2030 as the new target year for elimination of LF as a public health problem in 58 out of 72 countries (WHO, 2020).

Since 1974, various programs aimed at bringing onchocerciasis under control (Benton et al., 2002; Liese et al., 2010). In the Americas, four countries eliminated onchocerciasis. In 2025, Niger was the first country in Africa verified free of onchocerciasis (WHO, 2025b), bringing the WHO one step closer to reaching its target of elimination of onchocerciasis via interruption of transmission in 12 out of 38 countries by 2030 (WHO, 2020).

Despite numerous attempts, there are no approved vaccines for human filarial diseases (Kalyanasundaram et al., 2020; Zhan et al., 2022; Scheunemann et al., 2023). Prevention strategies thus rely on personal protective measures, e.g., long clothing, repellents, and insecticide-treated bed nets as well as vector control and preventive chemotherapy (PC) (Reimer et al., 2013; Alpern et al., 2016; WHO, 2020). Vector control includes chemical methods, such as the use of dichlorodiphenyltrichloroethane (DDT) to kill the infection-transmitting arthropods. While DDT was historically effective and widely used in the mid-20th century, its use has been largely prohibited since the Stockholm Convention in 2001 due to its harmful effects on wildlife and humans along with persistence in the environment (Turusov et al., 2002; Beard, 2006; Kabasenche and Skinner, 2014; Makgoba et al., 2024). As an alternative, *Wolbachia*-based vector control has emerged, which is discussed in 1.2.1.

PC, implemented via mass drug administration (MDA), is the WHO-recommended public health strategy to combat filarial diseases (Webster et al., 2014; WHO, 2020). MDA involves the large-scale distribution of drugs at regular intervals to entire population groups, regardless of infection status (certain limitations to this are discussed below), to reduce disease transmission. WHO launched MDA campaigns for LF in 2000 through GPELF (Ramaiah and Ottesen, 2014), while MDA campaigns for onchocerciasis had already been initiated in 1989 as part of the Onchocerciasis Control Programme (Sékétéli et al., 2002). To be successful, it has been suggested that MDA campaigns must cover at least 65% of the at-risk population for a minimum of five years (Stolk et al., 2006; WHO, 2024b). Therefore,

the donation of drugs through pharmaceutical partnerships has been instrumental in scaling up these campaigns, with many of the current donations being “open-ended”, i.e., continuing for as long as necessary (Bradley et al., 2021). Distribution of LF and onchocerciasis and the respective status of PC are shown in Fig. 3.

Drugs against filarial diseases are limited. Most commonly, ivermectin (IVM), diethylcarbamazine (DEC), and albendazole (ALB) are used (WHO, 2020; Ehrens et al., 2022). Satoshi Ōmura and William Campbell received the Nobel Prize in Physiology or Medicine in 2015 for their discovery of IVM, which led to a significant reduction in onchocerciasis and LF, as well as other helminth infections such as strongyloidiasis and ascariasis. Moreover, it has also been effective against ectoparasitic infections such as lice and scabies (Fox, 2006; Tambo et al., 2015).

MDA regimens for LF have evolved over time. Initially, DEC plus ALB or IVM plus ALB were used (Gyapong et al., 2005). The current WHO-recommended strategy for LF consists of annual triple therapy with IVM, DEC, and ALB (WHO, 2017b, 2020). As of 2023, 39 of 72 endemic countries required PC to eliminate LF transmission (Fig. 3A). Since the launch of MDA campaigns for LF, over 9.7 billion treatments have been delivered worldwide. In total, 33 countries have reached infection prevalence levels at which transmission is assumed to be unsustainable, 21 of which have been officially validated as having eliminated LF as a public health problem (WHO, 2024b, 2024d).

For onchocerciasis, a (bi-)annual MDA with IVM is recommended (WHO, 2020). As of 2023, PC remained necessary in 28 of 38 endemic countries to interrupt transmission (Fig. 3B) (WHO, 2024a). DEC, which had been used for about 40 years in onchocerciasis treatment, has been phased out due to severe adverse effects, particularly in individuals with high microfilarial loads (Fischer et al., 2017). The sudden death of large numbers of Mf can trigger severe inflammatory responses, leading to serious complications (Bryceson et al., 1977; Bird et al., 1980; Francis et al., 1985; Awadzi and Gilles, 1992). As soon as IVM as a safer alternative became available, the use of DEC was restricted (Awadzi and Gilles, 1992). Consequently, LF combination therapy with DEC is not recommended in countries co-endemic for onchocerciasis (Gyapong et al., 2005; WHO, 2017b).

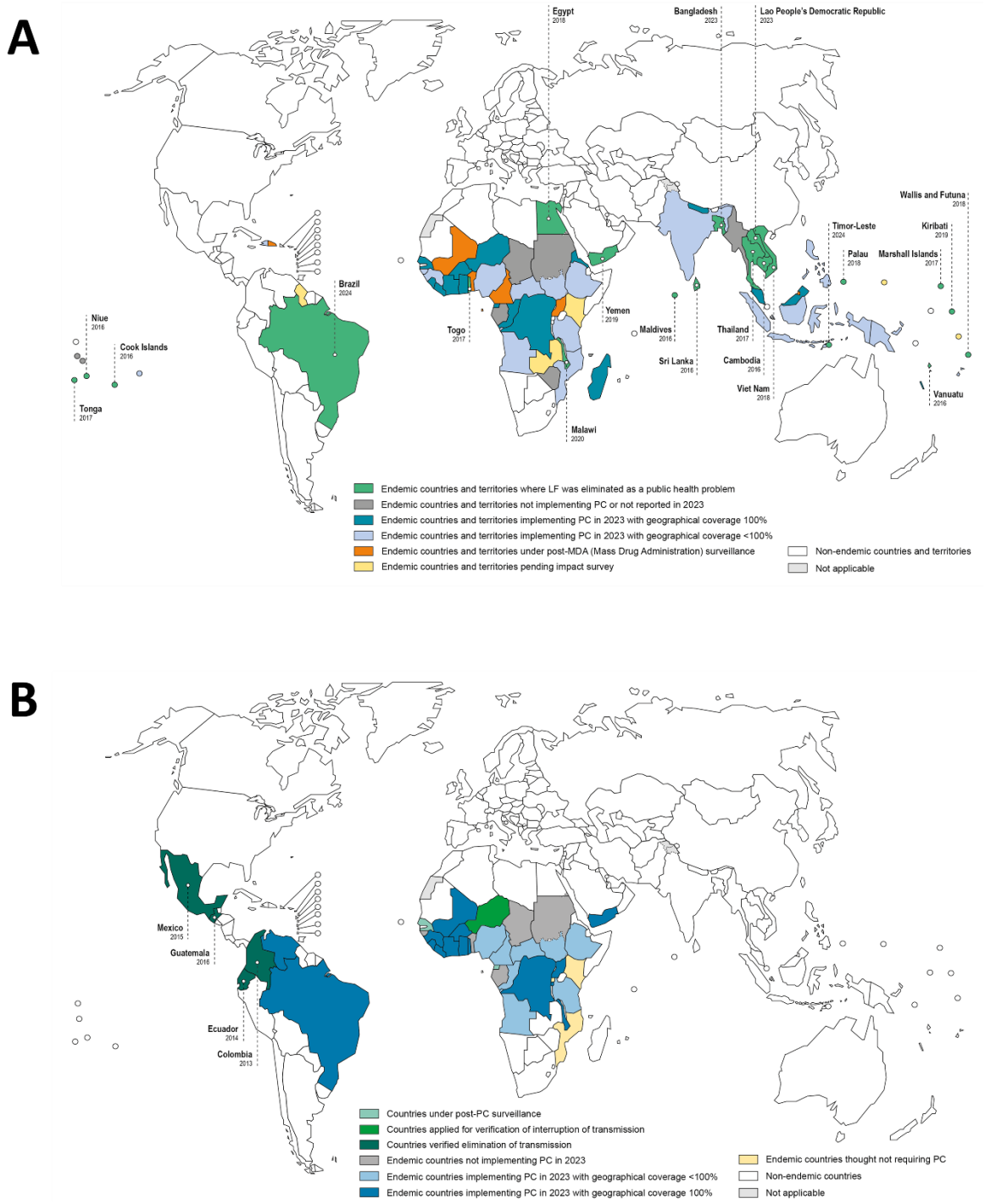

Figure 3. Distribution of lymphatic filariasis and onchocerciasis and status of preventive chemotherapy. The filarial diseases lymphatic filariasis and onchocerciasis are mainly targeted through preventative chemotherapy (PC). The maps indicate endemicity and the status of PC implementation in 2023 for lymphatic filariasis (**A**) and onchocerciasis (**B**). The images were adapted from WHO (2024e, 2024f).

A further challenge in PC for LF and onchocerciasis arises from co-endemicity with the filarial nematode *Loa loa*, as both IVM and DEC can cause severe adverse events in individuals with high *L. loa* Mf densities (Carme et al., 1991; Haselow et al., 2003; Taylor et al., 2010). IVM can be administered at *L. loa* Mf densities <8000 Mf/ml of blood, while DEC can be used at densities <2000 Mf/ml (Boussinesq, 2012; Davi et al., 2023). Treatment in cases of higher *L. loa* Mf densities can lead to progressive neurologic decline and encephalopathy, necessitating alternative strategies in these regions (Carme et al., 1991; Gardon et al., 1997; Bockarie and Deb, 2010). As such, "Test-and-Not-Treat" strategies, where individuals are screened for *L. loa* before treatment, may be employed (Kamgno et al., 2017; Lakwo et al., 2020; WHO, 2020). As a result of these contraindications, IVM cannot be used in areas where onchocerciasis is hypoendemic and loiasis is co-endemic (WHO, 2020). Similarly, the recommended triple therapy for LF with IVM, DEC, and ALB can only be implemented in countries where neither onchocerciasis nor loiasis are endemic. In regions where onchocerciasis is prevalent, IVM plus ALB is used, while in areas co-endemic for *L. loa*, treatment is restricted to ALB alone (WHO, 2017b). Table 1 summarizes the current WHO-recommended strategies for MDA.

Table 1. Mass drug administration for lymphatic filariasis and onchocerciasis.

Filarial disease	Co-endemicity	IVM[1]	DEC[2]	ALB[3]
Lymphatic filariasis	-	+	+	+
	Onchocerciasis	+		+
	Loiasis			+
Onchocerciasis	-	+		
	Loiasis	(+)*		

[1]ivermectin, [2]diethylcarbamazine, [3]albendazole
*IVM cannot be used safely in MDA settings in which onchocerciasis is hypoendemic and loiasis is co-endemic (WHO, 2020).

A major limitation of these drugs is their primarily microfilaricidal activity – i.e., the drugs kill the Mf but not the adult worms – necessitating long-term administration to suppress transmission. Since adult worms can persist for over a decade, stopping MDA poses a risk of reappearance even after prolonged control efforts, especially in hyperendemic regions

(Katabarwa et al., 2011). Further, reports of sub-optimal responses to IVM, DEC, and ALB raise concerns about emerging resistance (Cobo, 2016; Prichard, 2022; Tagboto and Orish, 2022). Macrofilaricidal (adult worm-killing) treatments that are also safe in regions co-endemic for *L. loa* are therefore needed to eliminate LF and onchocerciasis (Klarmann-Schulz et al., 2017; Hawryluk, 2020; Risch et al., 2024). IVM not only kills Mf but also temporarily suppresses embryogenesis in female worms (Richard-Lenoble et al., 2003; Fischer et al., 2023). ALB itself has limited antifilarial activity but may enhance the efficacy of other antifilarial treatments (Ehrens et al., 2022). DEC also predominantly acts against Mf (Hawking et al., 1950), but recent studies have demonstrated effects on adult worms (Verma et al., 2020; Williams et al., 2022).

The only safe macrofilaricidal therapy available is doxycycline (DOX), which targets the *Wolbachia* endosymbionts essential for filarial survival (see 1.2.2). DOX treatment not only reduces Mf levels and inhibits embryogenesis but also leads to a gradual decline in adult worm burden (Taylor et al., 2005b; Debrah et al., 2006; Hoerauf et al., 2008; Walker et al., 2015). However, the long treatment duration (4–6 weeks) and contraindications in pregnant and breastfeeding women as well as in children under eight years of age make DOX unsuitable for MDA (Hoerauf, 2008; Ehrens et al., 2022).

Currently, other macrofilaricidal drugs are being tested, encompassing compounds against *Wolbachia* such as rifampicin and corallopyronin A (CorA) (see 1.2.2) but also compounds directly acting against the nematode. The latter include moxidectin, emodepside, and oxfendazole in clinical development, and DNDi-6166 in preclinical development (Risch et al., 2024). These efforts are supported by partnerships and initiatives such as the Drugs for Neglected Diseases initiative (DNDi), the EU-funded project eWHORM (*enabling the WHO Road Map*), and the German Center for Infection Research (DZIF) (Ehrens et al., 2022; Risch et al., 2024; DNDi, 2025; DZIF, 2025a, b; eWHORM, 2025). Moxidectin was approved for onchocerciasis in 2018 and is now in clinical development for LF. Phase II clinical trials are currently ongoing for emodepside, while Phase I trials demonstrated that oxfendazole is safe and well tolerated in humans, with Phase II trials scheduled for 2025 (Risch et al., 2024). Table 2 provides an overview of approved drugs for LF and onchocerciasis, as well as those in (pre)clinical development, indicating whether they have macrofilaricidal effects and whether they act directly on adult worms or target *Wolbachia*.

Table 2. Antifilarial drugs for lymphatic filariasis and onchocerciasis, their targeted stage, and type of action.

	Direct[#]	Indirect (*Wolbachia*)^
Adult worm	DEC	DOX*
	IVM	Rifampicin*[1]
	ALB	CorA*[2]
	Moxidectin[1]	
	Emodepside*[1]	
	Oxfendazole*[1]	
	DNDi-6166[2]	
Microfilariae	DEC	
	IVM	
	Moxidectin[1]	

[#]diethylcarbamazine (DEC), ivermectin (IVM), albendazole (ALB)
^doxycycline (DOX), corallopyronin A (CorA)
*Drug has macrofilaricidal effect.
[1]Drug is in clinical development (moxidectin is already approved for onchocerciasis).
[2]Drug is in preclinical development.

Beyond pharmacological treatments, managing LF primarily relies on hygiene-based interventions to reduce disease progression and prevent secondary infections (Stocks et al., 2015; Ngenya et al., 2024). In cases of hydrocele, surgical intervention – hydrocelectomy – remains the primary treatment to relieve symptoms and improve quality of life (Betts et al., 2020; WHO, 2020, 2024b).

1.2 *Wolbachia*

Wolbachia were discovered in 1924 by Hertig and Wolbach (Hertig and Wolbach, 1924). Hertig later named them *Wolbachia pipientis* in honor of Wolbach and highlighting the mosquito in which they were discovered (*Culex pipiens*) (Hertig, 1936). These obligate intracellular bacteria belong to the order Rickettsiales and are closely related to *Ehrlichia*, *Anaplasma*, and *Neorickettsia* genera within the Alphaproteobacteria (Dunning Hotopp et

al., 2006). *Wolbachia* are found in a wide range of arthropods and filarial nematodes (Bandi et al., 1998; Taylor and Hoerauf, 2001; Fenn et al., 2006; Zug and Hammerstein, 2012). Only recently, they were discovered in other nematodes, namely in plant-parasitic nematodes (Haegeman et al., 2009; Brown et al., 2016; Wasala et al., 2019; Weyandt et al., 2022) and in an insect-parasitic nematode (Dudzic et al., 2022). Although the exact incidence is unclear, with varying infection frequency estimates of 22–66% in arthropods (Hilgenboecker et al., 2008; Zug and Hammerstein, 2012; Vancaester and Blaxter, 2023), *Wolbachia* are considered the most widespread endosymbionts in the animal kingdom (Kaur et al., 2021). To date, only a single *Wolbachia* species, *Wolbachia pipientis*, has been described; therefore, we will continue to refer to it simply as *Wolbachia*. Moreover, it has been argued that the *Wolbachia* strains of nematodes should not be grouped together with those of arthropods under *W. pipientis* (Pfarr et al., 2007). As of 2025, 19 phylogenetic clades, termed "supergroups" and named alphabetically, have been recognized, with supergroups A and B being the most common (Gerth et al., 2014; Lefoulon et al., 2020a; Rodrigues et al., 2023). Recently, two additional supergroups have been proposed (Sharma and Som, 2023). Each supergroup may contain different *Wolbachia* strains, which are named after their hosts, e.g., *w*Bm for the strain of *B. malayi* or *w*Mel for the one of *Drosophila melanogaster*.

In arthropods, *Wolbachia* are mainly facultative endosymbionts and they manipulate host reproduction to enhance their vertical transmission. These strategies include cytoplasmic incompatibility, parthenogenesis, feminization, and male killing (Werren, 1997; Stouthamer et al., 1999). In contrast, filarial nematodes rely on *Wolbachia* as obligate mutualists necessary for survival and embryogenesis (Bandi et al., 1999; Hoerauf et al., 1999). While *Wolbachia* themselves are not pathogenic, they play a critical role in human disease control strategies (Slatko et al., 2014). In vector control, *Wolbachia*-infected mosquitoes are released to reduce the transmission of vector-borne diseases (see 1.2.1). Conversely, in the treatment of filarial diseases, *Wolbachia* are targeted with antibiotics to indirectly deplete the filarial nematodes (see 1.1.2, Table 2, and 1.2.2).

Wolbachia are maternally inherited (Yen and Barr, 1971) and reside in vacuoles within host cells (Cho et al., 2011). They are pleomorphic, with a cell diameter typically ranging from 0.5 to 1.3 µm, although sizes from 0.2 µm up to 4.5 µm in length have been reported

(Hertig, 1936; Kozek, 2005; Ramírez-Puebla et al., 2015; Taylor et al., 2018). They usually retain a three-layered double-membrane structure, with the host-derived vacuolar membrane surrounding their cytoplasmic and outer membranes (Fischer et al., 2014). Although traditionally classified as Gram-negative due to their inability to retain the Gram stain, *Wolbachia*'s peptidoglycan (PGN) status is unclear (see 1.3.5). Once considered cell wall-less, *Wolbachia* are now classified as PGN-intermediate (Otten et al., 2018; Atwal et al., 2021). The term "diderm", simply referring to the presence of two membranes, may more accurately describe their envelope structure.

As obligate intracellular bacteria, *Wolbachia* possess a highly reduced genome, with sizes ranging from 0.55 megabases (Mb) (*Wolbachia* of *Howardula* sp. (Dudzic et al., 2022)) to 2.19 Mb (*Wolbachia* of *Ischnura elegans* (Vancaester and Blaxter, 2023)), and usually 1–1.5 Mb for facultative *Wolbachia* of arthropods and 0.86–1.1 Mb for obligate *Wolbachia* of filarial nematodes (Dudzic et al., 2022). They possess few transcriptional regulators, leading to the assumption that most of their genes are constitutively expressed (Wu et al., 2004; Foster et al., 2005). Transcriptome analyses have been challenging due to the difficulty of obtaining sufficient reads mapping to *Wolbachia*. A recent meta-analysis of seven RNA-seq datasets confirmed that *Wolbachia* largely lack global gene regulation, identifying fewer than 100 differentially expressed genes across various conditions (Chung et al., 2020).

Wolbachia have not been cultured extracellularly due to their dependence on host cells, which complicates genetic manipulation. However, studies have shown extracellular survival *in vitro* (Rasgon et al., 2006; Nevalainen et al., 2023) and *in vivo* (Dobson et al., 1999; Frydman et al., 2006; Fischer et al., 2011; Fischer et al., 2014), migration between tissues (Frydman et al., 2006; Fischer et al., 2011) and host species (Vavre et al., 1999; Ahmed et al., 2016; Vancaester and Blaxter, 2023; Ribeiro et al., 2025) as well as extracellular metabolic activity (Krafsur et al., 2020). *Wolbachia* are usually studied in whole organisms or insect cell lines, as nematode cell lines have not been successfully established yet (Slatko et al., 2014; Fallon, 2021).

1.2.1 *Wolbachia* in vector control

Vector-borne diseases account for over 17% of all infectious diseases and cause more than 700,000 deaths annually (WHO, 2017a). Mosquitoes are the primary vectors of human pathogens, transmitting diseases such as malaria, dengue, chikungunya, Zika, yellow fever, West Nile fever, and filariasis. The main vector species belong to the *Anopheles*, *Aedes*, and *Culex* genera (Song et al., 2022). The spread of these vectors is influenced by anthropogenic factors, including climate change (Semenza et al., 2016; Moretti et al., 2025). Notably, *Aedes albopictus*, the Asian tiger mosquito, is considered the most invasive mosquito species worldwide (Benedict et al., 2007). Targeting vectors rather than pathogens themselves is an effective strategy to reduce disease spread (Wilson et al., 2020).

Conventional vector control methods mostly rely on insecticides (chemical vector control). However, reduced efficacy, resistance development, negative effects on non-target species as well as toxic impact on humans and the environment have driven the search for alternative strategies (Ansari et al., 2014; Liu, 2015; Wang et al., 2021; Ahmad et al., 2024).

One alternative is *Wolbachia*-based vector control. An estimated 34% of Diptera, the order that includes mosquitoes, are infected with *Wolbachia* (Vancaester and Blaxter, 2023). In 1971, Yen and Barr hypothesized that *Wolbachia* are the etiological agent of cytoplasmic incompatibility, a phenomenon observed for decades, and later confirmed this hypothesis (Yen and Barr, 1971, 1973). Cytoplasmic incompatibility is a form of conditional sterility in which *Wolbachia*-infected males produce inviable offspring when mating with uninfected females or females carrying a different *Wolbachia* strain (Sinkins, 2004).

Two *Wolbachia*- and cytoplasmic incompatibility-based vector control strategies are deployed: the incompatible insect technique (IIT) and population replacement strategy (PRS). In IIT, by releasing large numbers of cytoplasmic incompatibility-inducing males, i.e., males infected with a different *Wolbachia* strain, the overall mosquito population is reduced (Crawford et al., 2020; Wang et al., 2021). Since male mosquitoes do not bite, and thus do not transmit the diseases, their release does not pose a public health or nuisance concern (Alphey et al., 2010). Due to *Wolbachia*'s maternal inheritance, the male *Wolbachia* strain does not spread in the population. However, accidental release of female *Wolbachia*-infected mosquitoes could lead to the stable establishment of the strain in the population (Xi et al., 2005; Soh et al., 2022). While this would render IIT ineffective, it could

instead shift the outcome from population suppression to suppression of virus transmission (as used in PRS) (Ross, 2021).

IIT has been implemented in several countries, including regions in the US (Mains et al., 2019; Crawford et al., 2020), China (Zheng et al., 2019), and Singapore (Lim et al., 2024). The resulting suppression of female mosquitoes reached up to 78% (Mains et al., 2019), 94% (Zheng et al., 2019; Crawford et al., 2020), and 91% (Lim et al., 2024), respectively.

In PRS, male and female mosquitoes infected with a *Wolbachia* strain that inhibits viral replication are introduced to replace native mosquito populations that efficiently transmit viruses to humans (Yen and Failloux, 2020; Wang et al., 2021). *Wolbachia* have been shown to reduce the vectoral capacity of mosquitoes by interfering with viral replication, potentially through competition for host cellular resources (Iturbe-Ormaetxe et al., 2011; Lindsey et al., 2018). For example, Edwards et al. (2023) found that *Wolbachia* alter cholesterol metabolism in mosquito cells, limiting viral replication.

PRS has already been employed in several regions to combat dengue. In Queensland, Australia, releases of *Wolbachia*-infected *Aedes aegypti* mosquitoes led to 96% reduction in dengue cases (Ryan et al., 2020; Ogunlade et al., 2023). Similar projects have been initiated in other countries, including Brazil (Pinto et al., 2021), Indonesia (Indriani et al., 2020), and Malaysia (Nazni et al., 2019), all of which have led to a reduction in dengue cases. These projects have mostly been coordinated by the World Mosquito Program. It started in 2011, has been operating in 14 countries so far, and has protected over 13 million people as of December 2024 (Montenegro et al., 2024; World Mosquito Program, 2025).

Wolbachia-based IIT and PRS offer promising alternatives for long-term vector management and the reduction of mosquito-borne diseases, while being much more environmentally friendly than chemical vector control (Montenegro et al., 2024). Moreover, unlike traditional insecticide-based approaches and IIT, PRS is self-sustaining (Ross, 2021; Bhattacharyya and Banerjee, 2024). Efficacy, safety, and sustainability of *Wolbachia*-based strategies have recently been reviewed by Moretti et al. (2025).

Wolbachia-based vector control strategies have also been explored for filariasis control but face key challenges such as the presence of multiple vector genera together with the presence of different filariae (Townson, 2002; Brelsfoard et al., 2008; Kambris et al., 2009;

Andrews et al., 2012). Consequently, the primary focus for filarial disease control remains on MDA (see 1.1.2) and anti-*Wolbachia* chemotherapy (see 1.2.2).

1.2.2 Anti-*Wolbachia* chemotherapy

Intracellular bacteria were first observed in various species of filarial nematodes in the 1970s, including *B. malayi*, *Brugia pahangi*, *O. volvulus*, *Dirofilaria immitis*, and *Dirofilaria repens* (McLaren et al., 1975; Vincent et al., 1975; Kozek and Marroquin, 1977). In the 1990s, these bacteria were identified as *Wolbachia* (Sironi et al., 1995; Werren, 1997; Bandi et al., 1998). The first antifilarial effects of tetracycline were reported by Bosshardt et al. (1993) in *B. pahangi*, and subsequent studies in the late 1990s revealed that the antibiotic inhibited filarial growth and fertility through depletion of *Wolbachia* (Genchi et al., 1998; Bandi et al., 1999; Hoerauf et al., 1999; McCall et al., 1999).

Most filarial nematodes that cause human disease, except *L. loa* (McGarry et al., 2003), harbor *Wolbachia* as endosymbionts in a mutualistic relationship essential for their development, survival, and reproduction (see 1.2.3). Depletion of *Wolbachia* via antibiotics disrupts embryogenesis, reduces Mf output, impairs worm development, and ultimately kills adult worms (Langworthy et al., 2000; Taylor et al., 2005a; Taylor et al., 2005b; Taylor et al., 2014). Since *Wolbachia* are evolutionarily distant from mammals, antibiotics that selectively target the endosymbiont offer a viable strategy for filarial disease treatment (Slatko et al., 2010).

Since the early 2000s, DOX has been used to treat filarial diseases (Hoerauf et al., 2000; Brouqui et al., 2001; Hoerauf et al., 2001; Hoerauf et al., 2003a; Hoerauf et al., 2003b). Although tetracycline was the first antibiotic to show antifilarial effects, DOX became the preferred treatment in humans due to its longer half-life, better tissue penetration, and fewer gastrointestinal side effects (Cunha et al., 1982; Lynn, 1996). Both antibiotics belong to the tetracycline class and work by inhibiting bacterial protein synthesis. They bind to the 30S ribosomal subunit of bacteria, preventing tRNA from attaching to the ribosome, thereby stopping protein production (Cunha et al., 1982). However, the clinical application of DOX for filarial infections is limited due to its prolonged treatment regimen and contraindications in certain population groups (see 1.1.2). These limitations preclude its

use in MDA programs, highlighting the need for alternative therapies with shorter treatment durations and fewer restrictions.

Therefore, different partners from academia, industry and non-profit organizations, including the anti-*Wolbachia* (A·WOL) consortium, are working on novel antibiotics for the treatment of filarial diseases (Johnston et al., 2021). For example, AWZ1066S was found superior to DOX *in vitro* but its clinical development was stopped after a Phase I study due to safety concerns (Devereux et al., 2024). Similarly, flubentylosin (formerly ABBV-4083), which had already entered Phase II, was superior to DOX in preclinical studies but was discontinued due to a lack of efficacy *in vivo* (Risch et al., 2024). Therefore, while there are currently some macrofilaricidal candidates in preclinical or clinical development (see 1.1.2), there are only two anti-wolbachial candidates in the pipeline, the novel antibiotic CorA and the repurposed antibiotic rifampicin (Ehrens et al., 2022; Risch et al., 2024). Both function as DNA-dependent RNA polymerase inhibitors (Calvori et al., 1965; Irschik et al., 1985). Since rifampicin is one of the most important drugs against tuberculosis, its use as an antifilarial drug would be limited to individual treatment rather than MDA to reduce the risk of antibiotic resistance in *Mycobacterium tuberculosis* (Risch et al., 2024).

CorA, derived from the myxobacterium *Corallococcus coralloides*, was identified as an effective anti-*Wolbachia* agent in 2012 (Schiefer et al., 2012). Since it binds to the switch region of the polymerase, no cross-resistance with rifampicin has been detected (Mukhopadhyay et al., 2008). In the *Litomosoides sigmodontis* jird model, a rodent model of filariasis using the filaria *L. sigmodontis* in *Meriones unguiculatus* (Mongolian jirds), a 14-day CorA treatment reduced *Wolbachia* levels by >99%, resulting in macrofilaricidal effects. Combining CorA with ALB shortened the treatment duration to seven days and increased adult worm mortality (Schiefer et al., 2020). Safety and toxicity tests *in vitro* and *in vivo* suggest that CorA is safe and non-toxic (Risch et al., 2024) and Phase I trials are scheduled for 2027 (K. Pfarr, personal communication). Beyond *Wolbachia*, CorA is also effective against other bacteria, including *Chlamydia trachomatis*, *Staphylococcus aureus*, rifampicin-resistant *S. aureus*, and *Neisseria gonorrhoeae* (Edwards et al., 2022; Krome et al., 2022).

The death of filarial worms and Mf triggers immune responses due to the release of parasite-derived antigens and, in *Wolbachia*-harboring species, bacterial components

(Taylor, 2003; Taylor et al., 2010; Risch et al., 2021). It was found that the severe inflammatory response upon death of adult worms and Mf (see 1.1.2) are linked to *Wolbachia* (Cross et al., 2001; Saint André et al., 2002; Hise et al., 2003; Taylor, 2003; Taylor et al., 2010). *Wolbachia*-induced inflammation is mediated via Toll-like receptor (TLR)-2 and TLR-6 signaling, triggering interferon and chemokine production. This can lead to lymphedema or hydrocele in LF and corneal opacity and blindness in onchocerciasis (Hise et al., 2007; Pfarr et al., 2009; Tamarozzi et al., 2011).

Anthelmintic treatment can intensify immune responses by causing rapid parasite death and subsequent bacterial release. Anti-*Wolbachia* therapy mitigates this risk by leading to sterility, i.e., inhibiting Mf production, and gradually depleting adult worms, which reduces the likelihood of severe adverse events (Cross et al., 2001; Hise et al., 2007; Pfarr et al., 2009; Taylor et al., 2010). Because *L. loa* does not harbor *Wolbachia*, targeting the endosymbiont presents a safer alternative in co-endemic regions where IVM and DEC treatment carry a risk of severe adverse events (Carme et al., 1991; Bockarie and Deb, 2010; Taylor et al., 2010) (see 1.1.2).

Additionally, DOX treatment has been shown to alleviate lymphedema pathology by reducing *Wolbachia*-induced inflammatory responses and vascular endothelial growth factors that regulate lymphangiogenesis (Debrah et al., 2006; Turner et al., 2006b; Mand et al., 2012). However, a recent study found no significant difference between intensified hygiene measures alone and those combined with DOX treatment in lymphedema patients (Ngenya et al., 2024). Possible explanations include an imbalance in lymphedema stages between the groups at baseline, a stricter hygiene protocol and less ongoing filarial transmission compared to earlier studies, and the fact that only one affected leg was included in the primary analysis. A subanalysis that included all affected legs at baseline showed that participants in the DOX-treated group were more likely to have a halt of progression than those in the placebo group (Ngenya et al., 2024).

The identification of *Wolbachia* as an essential endosymbiont in most filarial nematodes has led to the development of targeted anti-*Wolbachia* therapies (Hübner et al., 2022b). While DOX remains a valuable treatment, its limitations necessitate new macrofilaricidal agents. Continued research and clinical trials will be crucial in developing accessible and effective therapies to combat filarial infections.

1.2.3 *Wolbachia* symbiosis

Wolbachia have diverse symbiotic relationships with arthropods and nematodes. While *Wolbachia* mostly act as facultative reproductive parasites in arthropods, their relationship with filarial nematodes is obligate mutualistic (Slatko et al., 2010). In an insect-parasitic nematode, the 100% infection rate with a *Wolbachia* strain, which has the smallest wolbachial genome discovered to date, suggests a mutualistic relationship (Dudzic et al., 2022). As obligate intracellular bacteria, *Wolbachia* have undergone considerable genome reduction. Their genome size varies depending on the host and symbiotic lifestyle: *Wolbachia* genomes in arthropods typically range in size from 1–1.5 Mb, whereas genomes in filarial nematodes exhibit more extreme genome reduction, often falling below 1 Mb (Foster et al., 2005; Dudzic et al., 2022) (see 1.2). This reduction in size reflects the transition to obligate mutualists in filarial nematodes, resulting in increased host dependence. Nevertheless, the metabolic capacities of *Wolbachia* of arthropods and nematodes are still very similar (Foster et al., 2005).

Wolbachia have lost many biosynthetic pathways and are dependent on their hosts for essential compounds. They have only incomplete pathways for the synthesis of vitamins and cofactors such as nicotinamide adenine dinucleotide (NAD), biotin, lipoic acid, ubiquinone, folate, pyridoxal phosphate and coenzyme A (Slatko et al., 2010). They have also lost almost all genes required for amino acid biosynthesis, retaining only the pathway for *meso*-diaminopimelate (*m*-DAP), a component of PGN in Gram-negative bacteria (Foster et al., 2005) (see 1.3.1, 1.3.5). *Wolbachia* likely obtain amino acids via proteolysis of host proteins and import from the extracellular environment. The presence of various proteases encoded in their genome supports this hypothesis (Foster et al., 2005). It is of note that *Wolbachia* have retained key genes for the biosynthesis of lipid II, a precursor of PGN (see 1.3.5).

Despite intensive studies, the exact reason for the nematodes' dependence on *Wolbachia* remains unknown. *Wolbachia* are thought to provide essential nutrients: filarial nematodes lack the ability to synthesize several important metabolites, including riboflavin and, thus, flavin adenine dinucleotide (FAD), purines, pyrimidines, and heme, all of which *Wolbachia* can synthesize (Wu et al., 2004; Foster et al., 2005; Slatko et al., 2010). Although *Wolbachia* can synthesize nucleotides *de novo*, they do not have a salvage pathway, whereas the

opposite is true for the nematode host, confirming the assumption of nutrient interdependence. Transcriptomic analysis has shown that genes essential for reproduction, growth, and development of *B. malayi* are downregulated upon treatment with DOX, confirming the nematode's dependence on *Wolbachia* at the transcriptional level (Rao et al., 2012).

Experimental studies have suggested that the *Wolbachia* heme pathway might indeed be critical for *B. malayi* (Wu et al., 2009). Similarly, a comparison of gene expression in *L. sigmodontis* tetracycline-treated worms (without *Wolbachia*) and untreated worms (with *Wolbachia*) showed that the treated worms exhibited increased expression of nematode heme-binding globin and several heme- and riboflavin-containing respiratory chain components (Strübing et al., 2010). This led to the further study of heme biosynthesis inhibitors for anti-*Wolbachia* treatment (Lentz et al., 2013; Biney et al., 2023).

However, *Wolbachia* of the bovine parasite *Onchocerca ochengi* only has incomplete pathways for riboflavin and FAD (Lefoulon et al., 2020b) and many metabolic pathways are also absent in *L. loa* and *Acanthocheilonema viteae*, filarial nematodes without *Wolbachia*, calling into question the metabolic supply hypothesis (Bandi et al., 1998; McGarry et al., 2003; Slatko et al., 2010; Desjardins et al., 2013; Lentz et al., 2013).

Not all *Wolbachia* strains of arthropods are facultative in their hosts. *Wolbachia* are essential for reproductive processes in several arthropods (Dedeine et al., 2001; Timmermans and Ellers, 2009; Hosokawa et al., 2010; Miller et al., 2010). Furthermore, in bed bugs they provide B vitamins that are lacking in the blood diet. Bed bugs that have had the *Wolbachia* removed by antibiotic treatment are severely impaired in their development and reproduction (Hosokawa et al., 2010).

It has been suggested that the mutualistic effects of *Wolbachia* infection are not restricted to filarial nematodes and a few arthropod species, as their high prevalence in insects cannot be explained by reproductive manipulation alone (Hamm et al., 2014; Newton and Rice, 2020). The fitness of *Wolbachia* is closely linked to that of their host and any mutualistic benefits *Wolbachia* provide could enhance their own survival (Zug and Hammerstein, 2015). This possible dual nature of *Wolbachia* in arthropods as a beneficial symbiont and manipulative parasite has led to it being described as a potential "Jekyll and Hyde" endosymbiont (Jiggins and Hurst, 2011; Newton and Rice, 2020). Newton and Rice (2020)

propose experimental approaches for bees and flies using defined diets to test whether specific *Wolbachia* strains can supplement the metabolites predicted from their genomes.

1.3 Bacterial cell wall

The bacterial cell wall is essential for maintenance of cell shape and structural integrity of most bacteria. This, together with its absence from the eukaryotic realm, has made it a key area of research, being an ideal target for antibiotics (Schneider and Sahl, 2010) (see 1.3.3). It can be used for classification in two major groups via staining (Fig. 4) (Gram, 1884): Gram-positive bacteria possess a thick cell wall consisting of many layers of PGN (30–100 nm), which retain the crystal-violet stain. Gram-negative bacteria have a thinner cell wall with predominantly a single layer of PGN (<10 nm), which does not retain the stain and is surrounded by an outer membrane (Silhavy et al., 2010). The outer membrane usually contains lipopolysaccharides (LPS) in its outer leaflet (Kamio and Nikaido, 1976).

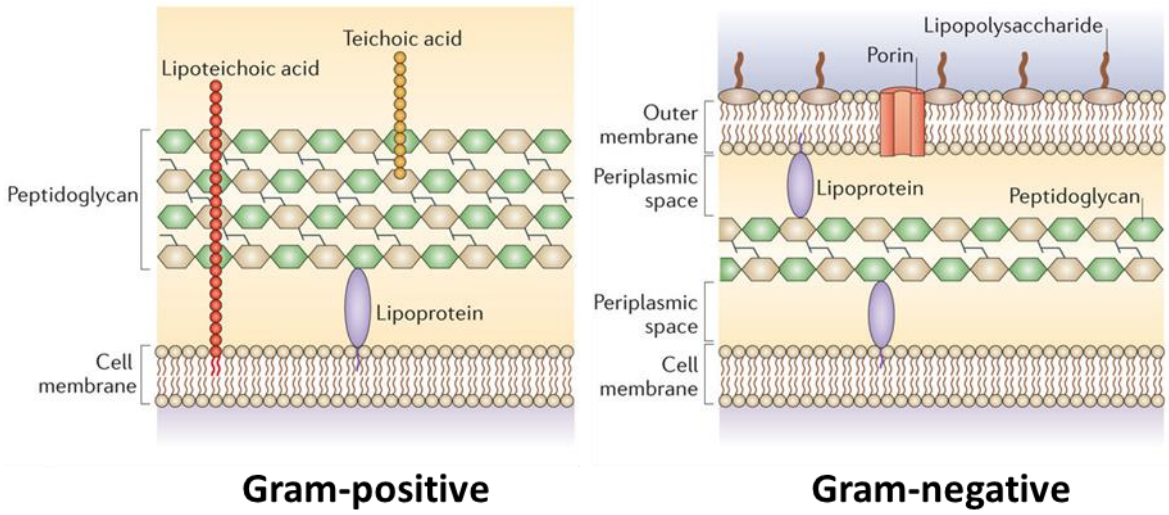

Figure 4. Cell wall structure of Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a cell wall composed of a thick peptidoglycan layer to which (wall) teichoic acids are linked, while lipoteichoic acids are anchored to the cell membrane. In contrast, Gram-negative bacteria have a cell wall consisting primarily of a thin peptidoglycan layer, located within the periplasmic space between the cell membrane (cytoplasmic membrane) and the outer membrane. The latter contains lipopolysaccharides in its outer leaflet and porins that facilitate passive diffusion of molecules. Lipoproteins are anchored to membranes in both Gram-positive and Gram-negative bacteria. The figure was adapted from Brown et al. (2015).

PGN, also known as murein, is synthesized via a highly conserved pathway and consists of glycan chains cross-linked by short peptides (see 1.3.1). It can also serve as an anchor for wall teichoic acids (in Gram-positive bacteria), proteins, and other surface structures (Brown et al., 2013; Guest and Raivio, 2016; Siegel et al., 2016).

Bacterial cell wall fragments play an important role in pathogenesis and disease, having immunostimulatory and cytotoxic properties (Boudreau et al., 2012; Jutras et al., 2019). Pattern recognition receptors which recognize PGN are found in diverse eukaryotes including flies, mice, humans as well as plants (Chaput and Boneca, 2007; Sorbara and Philpott, 2011; Gust, 2015). Humans have four secreted PGN recognition proteins that directly bind extracellular PGN (Liu et al., 2000; Lu et al., 2006). Intracellular PGN is detected by two intracellular pattern recognition receptors, nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 (Chamaillard et al., 2003; Inohara et al., 2003).

1.3.1 Peptidoglycan biosynthesis in free-living Gram-negative bacteria

PGN biosynthesis is a highly conserved multi-step process beginning in the cytoplasm and ending in the periplasm, after passing through the cytoplasmic membrane (Fig. 5). The majority of proteins involved in this pathway are members of either the murein (Mur) or penicillin-binding protein (PBP) families (Typas et al., 2011).

The first precursor, uridine diphosphate *N*-acetylglucosamine (UDP-Glc*N*Ac), is synthesized from fructose-6-phosphate by the sequential action of GlmS, GlmM, and GlmU (Badet et al., 1988; Mengin-Lecreulx and van Heijenoort, 1994, 1996). The first committed step of PGN synthesis is the transfer of an enolpyruvate residue from phosphoenolpyruvate to UDP-Glc*N*Ac, catalyzed by the transferase MurA. The following reduction of the enolpyruvate residue to D-lactate is catalyzed by MurB and yields UDP-*N*-acetylmuramic acid (UDP-Mur*N*Ac) (Gunetileke and Anwar, 1966; Bugg and Walsh, 1992). In the next phase, a series of ATP-dependent ligases (MurC, MurD, MurE, and MurF) catalyze the stepwise addition of amino acids forming a pentapeptide chain attached to UDP-Mur*N*Ac, creating UDP-Mur*N*Ac-pentapeptide. Specifically, MurC adds the first amino acid, commonly L-alanine (L-Ala), MurD adds D-glutamate (D-Glu) at the second position, and

MurE introduces a diamino acid, typically *m*-DAP in Gram-negative bacteria (Barreteau et al., 2008). The fourth and fifth amino acids are added as a dipeptide, usually D-Ala-D-Ala, synthesized by the D-Ala:D-Ala ligase (Ddl) and incorporated by MurF (Neuhaus and Struve, 1965; Duncan et al., 1990). Bacteria possess racemases to form the unusual D-amino acids (Radkov and Moe, 2014).

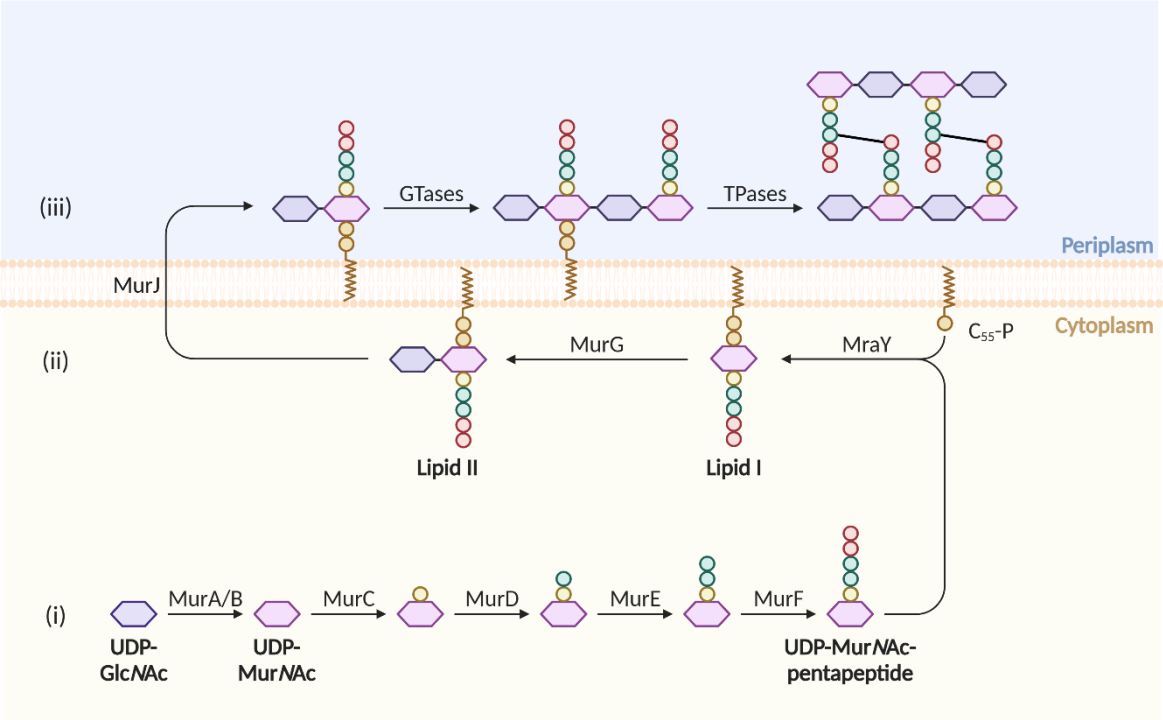

Figure 5. Peptidoglycan biosynthesis in Gram-negative bacteria. (i) Peptidoglycan biosynthesis is initiated in the cytoplasm, where MurA and MurB catalyze the conversion of UDP-Glc*N*Ac (uridine diphosphate *N*-acetylglucosamine) to UDP-Mur*N*Ac (UDP-*N*-acetylmuramic acid). A pentapeptide chain (circles) is added to UDP-Mur*N*Ac through the sequential action of MurC, MurD, MurE, and MurF, with the latter adding the terminal D-Ala-D-Ala dipeptide. (ii) MraY transfers Mur*N*Ac-pentapeptide onto the membrane carrier undecaprenyl phosphate (C_{55}-P), forming Lipid I. MurG then adds Glc*N*Ac, generating Lipid II, which is flipped across the membrane by MurJ. (iii) In the periplasm, transglycosylases (GTases) polymerize glycan strands, while transpeptidases (TPases) cross-link peptide side chains, forming the mature peptidoglycan layer. The common 4,3-cross-links are shown, which are formed under cleavage of the terminal D-Ala (red circle) from the donor pentapeptide. Adapted from BioRender (2020) with BioRender.com.

Following pentapeptide synthesis, the UDP-Mur*N*Ac-pentapeptide moiety is transferred to the lipid carrier undecaprenyl phosphate (C_{55}-P) by the enzyme MraY, generating lipid I (Ikeda et al., 1991). Next, MurG ligates Glc*N*Ac to lipid I, forming lipid II, the lipidated

disaccharide-pentapeptide and ultimate cell wall precursor (Chen et al., 2002; Bouhss et al., 2008). Lipid II is then translocated across the cytoplasmic membrane by a flippase to the outer face of the membrane, where it becomes incorporated into the growing PGN mesh (den Blaauwen et al., 2008). The identification of the lipid II flippase has been the subject of significant controversy, with three candidates: FtsW, RodA, and MurJ. FtsW and RodA, both members of the shape, elongation, division, and sporulation (SEDS) family, were first proposed as lipid II flippases because they are membrane proteins involved in PGN synthesis (Matsuhashi, 1994; Höltje, 1998). MurJ was later suggested as a flippase based on its essential role in *Escherichia coli* (Ruiz, 2008). *In vitro* assays demonstrated that FtsW could translocate lipid II, supporting its potential role as a flippase (Mohammadi et al., 2011). However, more recent evidence, including *in vivo* biochemical assays, confirms that MurJ is the primary lipid II flippase, while FtsW and RodA are primarily involved in glycan polymerization during PGN synthesis (Sham et al., 2014; Meeske et al., 2015; Ruiz, 2016) (see 1.3.2). After the release of the PGN from the lipid carrier, C_{55}-PP is recycled. Therefore, it is dephosphorylated to C_{55}-P, which is flipped back to the cytoplasmic side of the membrane (Storm and Strominger, 1973).

In the periplasm, the synthesis of the PGN mesh from lipid II involves two critical biosynthetic processes: transglycosylation and transpeptidation. Transglycosylation, catalyzed by glycosyltransferases (GTases), involves the polymerization of lipid II by forming β-(1,4)-glycosidic bonds between Glc*N*Ac and Mur*N*Ac residues. This reaction extends the glycan strands, creating the backbone of PGN (van Heijenoort, 2001). Following this, transpeptidation, mostly catalyzed by DD-transpeptidases (DD-TPases), cross-links the peptide stems attached to the Mur*N*Ac residues (Vollmer et al., 2008a). Both reactions are mostly catalyzed by PBPs, named after their capacity to covalently bind penicillin (Suginaka et al., 1972). Class A PBPs (aPBPs) are bifunctional GTase-TPases, while class B PBPs (bPBPs) are monofunctional TPases, requiring separate enzymes for GTase activity. This function is carried out by monofunctional GTases (Di Berardino et al., 1996) and the SEDS family proteins, RodA and FtsW (Cho et al., 2016; Meeske et al., 2016; Emami et al., 2017; Taguchi et al., 2019) (see 1.3.2). It was long assumed that bacteria with PGN need at least one aPBP for glycan polymerization. However, the joint action of a SEDS protein and a bPBP allows

for PGN synthesis without aPBPs (Reichmann et al., 2019; Taguchi et al., 2019; Sjodt et al., 2020; Atwal et al., 2021) (see 1.3.4).

DD-TPases form covalent bonds between the D-Ala at position 4 of one peptide stem and a diamino acid at position 3 of another (4,3-cross-links), providing the structural strength of the cell wall (Vollmer et al., 2008a). The degree of cross-linking is thereby regulated by DD-carboxypeptidases (DD-CPases), class C PBPs, which remove the terminal D-Ala from the stem pentapeptide required for DD-TPase activity (de Pedro et al., 1980; Ghosh et al., 2008). However, cross-links between glycan chains can also be formed by LD-transpeptidases, which are able to process tetrapeptide stems, creating 3,3-cross-links (Mainardi et al., 2008). While lipid II synthesis is highly conserved, the final PGN structure varies among bacteria due to differences in cross-linking type (4,3- vs. 3,3-cross-links) and degree (percentage of cross-links) (Snowden and Perkins, 1990; Quintela et al., 1995).

PGN is not a static structure but is turned over and remodeled by PGN hydrolases, such as endopeptidases, CPases, amidases, and muramidases, during cell growth and division (Höltje, 1998; Vollmer et al., 2008b). Endopeptidases and CPases cleave the amide bonds between amino acids in PGN or its soluble fragments, amidases cut the bond between the sugar and the peptide moieties, and muramidases, such as lysozymes and lytic transglycosylases, cleave the glycosidic bond between Glc*N*Ac and Mur*N*Ac (Park and Uehara, 2008; Vollmer et al., 2008b). The resulting PGN fragments are recycled by transporting them into the cytoplasm and trimming them for use in lipid II biosynthesis (Park and Uehara, 2008; Vollmer et al., 2008b). Recycling is advantageous not only with respect to these metabolites but also in terms of minimizing the exposure to immune responses of a host (Boudreau et al., 2012).

1.3.2 Elongasome and divisome

The PGN layer usually forms a continuous, mesh-like structure that surrounds the bacterial cell. This rigid yet dynamic macromolecule, known as the sacculus, maintains cell integrity by withstanding internal turgor pressure while allowing for controlled growth and remodeling (Höltje, 1998; Garde et al., 2021). To achieve this, bacteria rely on the coordinated action of two distinct biosynthetic machineries, the elongasome and divisome,

which mediate cell wall expansion and remodeling during growth and division, respectively (den Blaauwen et al., 2008; Szwedziak and Löwe, 2013).

Late-stage PGN-synthesizing enzymes play essential, cell cycle-specific functions, primarily acting during either cell elongation or division (Scheffers and Pinho, 2005). Among these, the SEDS proteins RodA and FtsW have been identified as key players in PGN polymerization, interacting with bPBPs to coordinate bacterial cell wall biosynthesis (see 1.3.1). In *E. coli*, RodA interacts with the TPase PBP2, while FtsW interacts with the TPase PBP3 (also known as FtsI) (Mercer and Weiss, 2002; Rohs et al., 2018). It is now evident that the RodA-PBP2 and FtsW-PBP3 pairs constitute the core PGN-synthesizing machineries of the elongasome and divisome, respectively (de Pedro et al., 2001; Datta et al., 2006; den Blaauwen et al., 2008; Uehara and Park, 2008; Fraipont et al., 2011). This functional division is reflected in the effects of antibiotics targeting these complexes: when treating rod-shaped *E. coli* cells with a PBP2-specific antibiotic, e.g., mecillinam, or upon inactivation of PBP2 or RodA they become round-shaped (Matsuzawa et al., 1973; Waxman and Strominger, 1983). In contrast, when treating with a PBP3-specific antibiotic, e.g., piperacillin, or upon inactivation of PBP3, cell division is inhibited and the cells become elongated (Spratt, 1977; Botta and Park, 1981). This refined model of PGN synthesis challenges the long-standing view that aPBPs are the central players in bacterial cell wall expansion (Cho et al., 2016; Emami et al., 2017; Straume et al., 2021).

As multiprotein complexes, the elongasome and divisome rely on additional regulatory factors to ensure spatial and temporal control over PGN biosynthesis. While their composition varies across bacterial species, their core components appear to be conserved (Fig. 6). The elongasome is primarily responsible for lateral cell wall biosynthesis, enabling elongation in rod-shaped bacteria. Its spatial organization is regulated by the bacterial actin homolog MreB, which forms cytoplasmic, membrane-associated filaments that coordinate the activity of the RodA-PBP2 complex, along with MreC, MreD, and RodZ (Jones et al., 2001; van den Ent et al., 2001; Bendezú et al., 2009; Liu et al., 2020; Garde et al., 2021; Perez et al., 2024). While MreB-dependent elongasome activity is essential for rod-shaped bacteria, cocci generally lack MreB and mainly grow via their division septum. In these bacteria, FtsZ likely serves as the sole organizer of PGN biosynthesis (Margolin, 2009). Mutation or inhibition of MreB leads to a loss of rod shape, resulting in a spherical

morphology (Normark, 1969; Wachi and Matsuhashi, 1989; Karczmarek et al., 2007; Jiang et al., 2011). MreB co-localizes with MreC, which was shown to play a key role in regulating elongation (Sjodt et al., 2020; Martins et al., 2021). Consequently, almost all species that have MreB also have MreC (exceptions include *Wolbachia*) (Alyahya et al., 2009; Margolin, 2009). There are exceptions of bacteria that have lost MreB and/or FtsZ and have evolved distinct mechanisms to maintain their shape and for cell division, respectively (see 1.3.4). In newly divided cells, PGN is predominantly synthesized along the sidewall, resulting in elongation. For cell division, the site of PGN synthesis changes from sidewall to division septum, where the divisome takes over (Lleo et al., 1990).

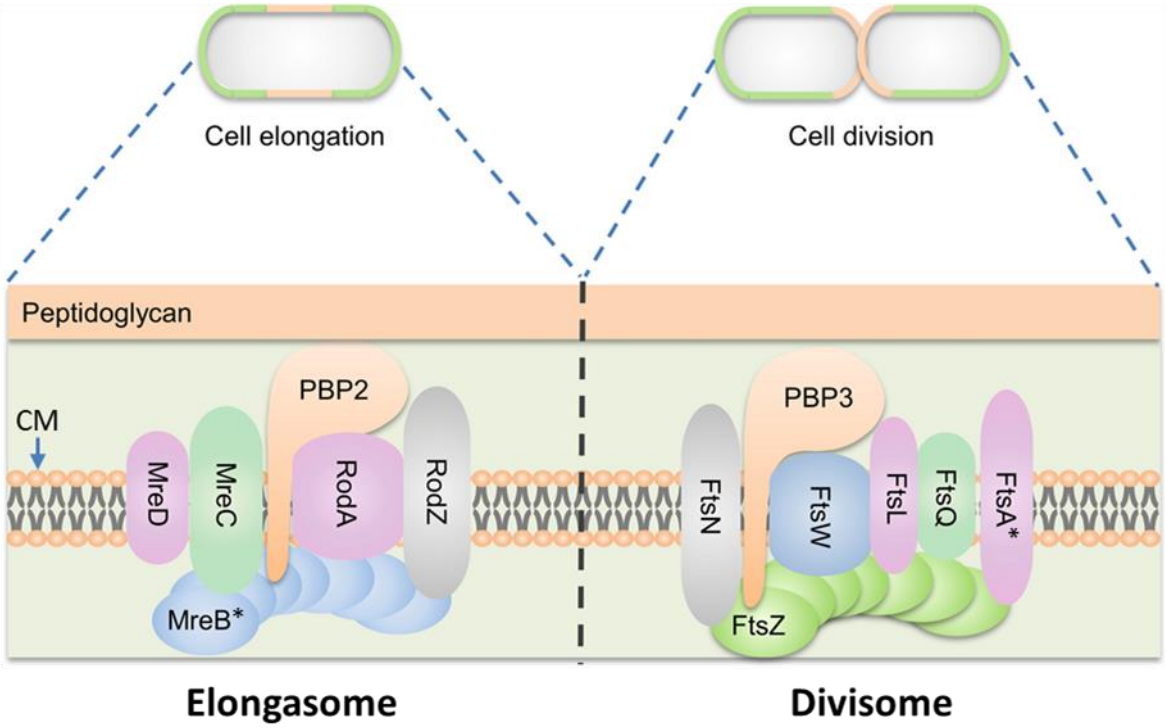

Figure 6. Core components of the elongasome and divisome. Rod-shaped bacteria coordinate cell wall biosynthesis through two distinct processes: elongation and septum formation. The elongasome, responsible for lateral cell wall elongation, includes the actin-like protein MreB, MreC, MreD, RodZ, RodA, and PBP2. The divisome, which directs cell division, is centered around the tubulin-like protein FtsZ, which recruits key division proteins, including FtsA, FtsL, FtsN, FtsQ, FtsW, and PBP3. For simplicity, additional cell-division proteins are not depicted. The involved proteins are either membrane-associated (*) or integral membrane proteins of the cytoplasmic membrane (CM) apart from FtsZ, which is cytoplasmic. The figure was adapted from Huo et al. (2020).

The divisome governs septal PGN synthesis and cell division. It is assembled around the bacterial tubulin homolog FtsZ, which polymerizes into a cytokinetic ring, the Z ring, at the site of cell division (Bi and Lutkenhaus, 1991; Ma et al., 1996; Margolin, 2005; Goehring et al., 2006; Strauss et al., 2012). FtsZ, together with FtsA, recruits additional essential proteins such as FtsK, FtsQ, FtsL, FtsB, and FtsN to coordinate the activity of the FtsW-PBP3 complex (Margolin, 2005; den Blaauwen et al., 2008; Villanelo et al., 2011; den Blaauwen and Luirink, 2019; Egan et al., 2020). Notably, in *E. coli*, the divisome consists of 12 essential proteins and more than 30 proteins in total (Pichoff et al., 2018; Garde et al., 2021; Radler and Loose, 2024).

For cell division, bacteria also need to break the bonds in the existing PGN sacculus, making autolysins essential for growth (Scheffers and Pinho, 2005). In *E. coli*, as much as 45% of the sacculus is removed during each cell cycle (Goodell, 1985). Since the poles of the cell are stable, more than 60% of the PGN of the sidewall is removed (Uehara and Park, 2008). The resulting degradation products are recycled and made available for the synthesis of new PGN (Goodell, 1985; Park and Uehara, 2008) (see 1.3.1).

1.3.3 Cell wall biosynthesis inhibitors

Since the bacterial cell wall is an essential structure that provides shape, mechanical stability, and protection against osmotic stress, its biosynthesis is a critical vulnerability exploited by numerous antibiotics, with lipid II representing a particular “Achilles’ heel” (Breukink and de Kruijff, 2006; Schneider and Sahl, 2010). Because PGN is absent from eukaryotes, it provides an attractive target for antibiotic therapy (Dörr et al., 2019). The discovery of penicillin, the first antibiotic and a cell wall biosynthesis inhibitor, by Alexander Fleming in 1928 marked the beginning of antibiotic therapy, leading to a golden age of antibiotic discovery in the mid-20th century (Fleming, 1929; Hutchings et al., 2019). Among the diverse classes of antibiotics discovered, a significant proportion are cell wall biosynthesis inhibitors, which constitute 50% of prescribed antibiotics (Schneider and Sahl, 2010; Hutchings et al., 2019).

Cell wall biosynthesis inhibitors target many steps of the PGN biosynthesis pathway (Fig. 7). Fosfomycin (FOS) inhibits MurA, the enzyme catalyzing the first committed step of PGN

precursor synthesis (Hendlin et al., 1969; Kahan et al., 1974). D-cycloserine, a structural analogue of D-Ala, inhibits alanine racemases and Ddl (Strominger et al., 1960). Five classes of nucleoside antibiotics, e.g., muraymycins and tunicamycins, target MraY, which catalyzes the first membrane-bound step in lipid II biosynthesis (Bugg et al., 2006). Among the most clinically significant inhibitors are the β-lactams, including penicillins, cephalosporins, carbapenems, monobactams, and penems, which block transpeptidation by inhibiting PBPs (Tipper and Strominger, 1965; Lima et al., 2020). For inhibition of transglycosylation, there is only one known potent antibiotic family, the moenomycins (Ostash and Walker, 2010).

In addition to the aforementioned cell wall biosynthesis inhibitors that target enzymes involved in PGN biosynthesis, others directly bind to target structures. Many antibiotics from diverse classes bind to lipid II (Grein et al., 2019). Glycopeptide antibiotics, such as vancomycin, function by binding to the D-Ala-D-Ala termini of lipid II, sterically hindering transglycosylation and transpeptidation (Reynolds, 1989). Lantibiotics, e.g., nisin, but also defensins, e.g., plectasin, also bind lipid II, with nisin and plectasin mostly binding the pyrophosphate moiety (Brötz et al., 1998; Hsu et al., 2004; Asaduzzaman and Sonomoto, 2009; Schneider et al., 2010; Grein et al., 2019; Jekhmane et al., 2024). Bacitracin binds to C_{55}-PP, thereby inhibiting its dephosphorylation and, thus, the recycling of the lipid carrier (Siewert and Strominger, 1967). More recently, the depsipeptide teixobactin has emerged as a promising new cell wall biosynthesis inhibitor that binds to the pyrophosphate moiety of not only lipid II but also of C_{55}-PP, and of lipid III, the wall teichoic acid precursor in Gram-positive bacteria (Ling et al., 2015).

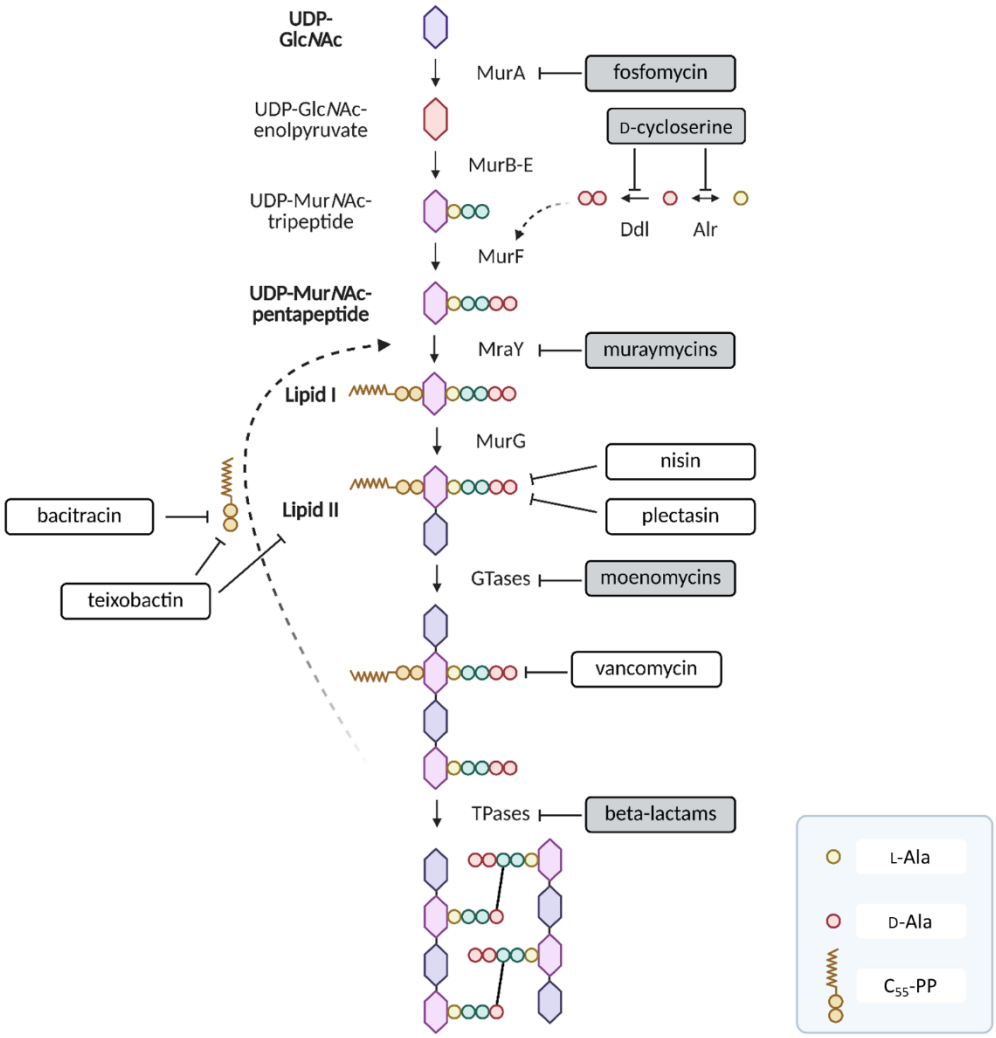

Figure 7. Cell wall biosynthesis inhibitors. Overview of peptidoglycan biosynthesis with selected cell wall biosynthesis inhibitors, binding to enzymes (grey boxes) and target structures (white boxes). Created with BioRender.com.

Inhibition of cell wall biosynthesis leads to weakened cell walls due to an imbalance between PGN biosynthesis and degradation, usually resulting in cell lysis (Rogers, 1967; Tomasz, 1974; Dörr, 2021). While the interactions of cell wall biosynthesis inhibitors with their targets have been studied in detail, comparatively little is known about the downstream cellular effects (Schneider and Sahl, 2010; Grein et al., 2019). Since cell wall biosynthesis must be tightly coordinated with cell elongation and division (see 1.3.2), its inhibition can disrupt these processes. Apparently, cell wall biosynthesis inhibitors induce delocalization of spatially organized enzymes, other components of the cell wall, and

divisomes (Grein et al., 2019). In rod-shaped bacteria, inhibition of cell division typically results in filamentation due to continued elongation without septation, whereas cocci often exhibit abnormal enlargement (Schneider and Sahl, 2010).

However, inhibition of cell wall biosynthesis does not always lead to immediate cell lysis. Under osmoprotective conditions, they can lead to the formation of cell wall-deficient L-forms (Leaver et al., 2009; Errington, 2017). Intracellular pathogens, such as Chlamydia, can form large, undividing cells, referred to as aberrant bodies (ABs) in Chlamydiae, upon exposure to these inhibitors (Atwal et al., 2017; Kozusnik et al., 2024). These morphological changes allow for persistence and survival under antibiotic pressure, thus complicating treatment strategies (Panzetta et al., 2018).

Bacteria have developed a plethora of resistance mechanisms such as antibiotic-degrading enzymes, target modification, and drug efflux, necessitating the search for novel antibiotics. Antibiotic resistance is unavoidable, but largely driven by misuse and overuse of antibiotics, with resistance known for each clinically used antibiotic to date (Laxminarayan et al., 2013; Wright, 2016; Uddin et al., 2021). In 2019, an estimated 4.95 million deaths were associated with antimicrobial resistance (AMR), of which an estimated 1.27 million were directly attributed to AMR (Antimicrobial Resistance Collaborators, 2022). Antibiotic resistance mechanisms to cell wall biosynthesis inhibitors are reviewed by Nikolaidis et al. (2014) and Sarkar et al. (2017). These include mutations, e.g., a point mutation in MurA of Chlamydia, rendering them resistant to FOS, and antibiotic-degrading enzymes such as beta-lactamases, degrading beta-lactams (McCoy et al., 2003; Nikolaidis et al., 2014). The outer membrane of Gram-negative bacteria already makes them resistant to large antibiotics such as glycopeptides, since these compounds cannot cross the membrane to reach their targets (Sarkar et al., 2017).

Understanding the mechanisms and cellular effects of cell wall biosynthesis inhibitors remains critical for developing new strategies to combat bacterial infections, especially in the face of rising antibiotic resistance. The continued exploration of novel compounds and resistance mechanisms will be essential in the ongoing fight against bacterial pathogens.

1.3.4 Peptidoglycan in obligate intracellular bacteria

Bacteria have adapted to a variety of environments, with one of the most specialized niches being the interior of eukaryotic cells. Some bacteria, known as facultative intracellular bacteria, can switch between intracellular and extracellular lifestyles, while obligate intracellular bacteria have lost the ability to replicate outside a host cell. These obligate intracellular bacteria replicate either in the host cell's cytoplasm or within specialized vacuoles (Otten et al., 2018; Clemente et al., 2023). Due to their small size and inability to be cultured outside host cells, obligate intracellular bacteria were sometimes misclassified, as in the case of *Chlamydia*, which was considered a virus until 1966 (Moulder, 1966). The first obligate intracellular bacteria to be identified were *Rickettsia* species, discovered and described by Ricketts in the early 1900s (Walker, 2004).

A shared trait of obligate intracellular bacteria is their highly reduced genome, reflecting their adaptation to the stable intracellular environment and their dependence on host-derived nutrients (Stepkowski and Legocki, 2001). While this lifestyle protects them from many extracellular immune defenses, it places strong selective pressure on their PGN (Otten et al., 2018; Atwal et al., 2021). Two key factors likely drive PGN reduction or modification in these bacteria: the intracellular environment, which provides osmotic stability and reduces the necessity for a rigid cell wall, and the immune evasion, as host cells possess PGN receptors that trigger immune responses (Chaput and Boneca, 2007; Sorbara and Philpott, 2011; Otten et al., 2018; Atwal et al., 2021). However, in some cases, PGN-related genes are selectively retained, possibly to support cell division or maintain structural maintenance (Otten et al., 2018; Atwal et al., 2021).

Examples of PGN-negative obligate intracellular bacteria include *Ehrlichia* and *Anaplasma phagocytophilum* (Lin and Rikihisa, 2003). They have lost all genes required for generation and recycling of C_{55}-P, lipid II biosynthesis, and PGN assembly. The only exceptions are MurB and MurJ, which are present in *A. phagocytophilum* (Otten et al., 2018). In contrast, the obligate intracellular *Coxiella burnetii* and *Rickettsia* species possess a classical PGN and have conserved all genes for lipid II biosynthesis and PBPs, including an aPBP, putative flippases, and at least one SEDS protein for PGN assembly (Otten et al., 2018).

Based on the occurrence of genes involved in PGN metabolism, Otten et al. (2018) identified a group of "PGN-intermediate" bacteria. These include Chlamydiae

(Chlamydiota), *Orientia tsutsugamushi*, *Wolbachia* (see 1.3.5), and *Anaplasma marginale*. MurA to MurG and MraY are present in these PGN-intermediate bacteria. However, biosynthetic proteins for *m*-DAP and UDP-Glc*N*Ac are not always present. At least one ortholog of a SEDS protein (FtsW/RodA) and a bPBP (PBP2/PBP3) are present in these PGN-intermediate species, while aPBPs are absent.

Chlamydiae were long thought to be PGN-negative, though they were known to be sensitive to cell wall biosynthesis inhibitors, i.e., beta-lactams, D-cycloserine, and bacitracin. This paradox was referred to as the "chlamydial anomaly" (Moulder, 1993; Chopra et al., 1998). Chlamydiae do not possess any aPBPs that were thought to be necessary for PGN biosynthesis. However, they do have bPBPs and SEDS proteins (Meeske et al., 2016; Cox et al., 2020). The same was observed for all other PGN-intermediate bacteria (Otten et al., 2018; Atwal et al., 2021). Thus, bPBPs and SEDS proteins were found to be more conserved than aPBPs and seem to constitute the basic cell wall-building machinery (Meeske et al., 2016; Atwal et al., 2021) (see 1.3.2). Transglycosylation is likely conferred by the SEDS protein(s) or from a yet unknown GTase (Atwal et al., 2021).

Since Chlamydiae lack FtsZ and cannot replicate without PGN synthesis, it was suggested that PGN or its precursor lipid II are instead required for chlamydial division (Chopra et al., 1998; Margolin, 2005; McCoy and Maurelli, 2006; Henrichfreise et al., 2009; Skilton et al., 2009). This could be confirmed through highly sensitive MS analysis and D-amino acid dipeptide probes and click chemistry, solving the chlamydial anomaly (Pilhofer et al., 2013; Liechti et al., 2014; Jacquier et al., 2015b): some environmental isolates possess complete PGN sacculi (Pilhofer et al., 2013), while the human pathogenic *C. trachomatis* has no PGN sacculus but a transient PGN ring structure which localizes to the division septum (Liechti et al., 2014; Packiam et al., 2015; Liechti et al., 2016).

Beta-lactams usually act bactericidal; however, in *Chlamydia*, they act bacteriostatically, leading to ABs (Storey and Chopra, 2001; Skilton et al., 2009). These enlarged *Chlamydia* are often polyploid (Lambden et al., 2006; Skilton et al., 2009). A similar phenomenon has been observed in the related *Waddlia chondrophila*, a member of Chlamydiae. It was thought to be PGN-negative as well, however cell wall biosynthesis inhibitors induced ABs (Jacquier et al., 2014; Scherler et al., 2020). PGN isolation and ultra-performance liquid

chromatography-mass spectrometry (UPLC-MS) analysis has then revealed PGN of *Waddlia chondrophila* (Jacquier et al., 2019).

Like Chlamydiae and *Wolbachia*, *O. tsutsugamushi* was historically thought to lack PGN (Amano et al., 1987), despite the presence of a nearly complete PGN biosynthesis pathway (Cho et al., 2007; Min et al., 2008; Nakayama et al., 2008). In the past decade, it was found that *O. tsutsugamushi*, while resistant to beta-lactams, is sensitive to D-cycloserine and FOS, with FOS inducing enlarged cells. Staining with fluorescent probes revealed a PGN-like structure (Atwal et al., 2017).

An overview of the key genes in the PGN biosynthesis pathway of the aforementioned obligate intracellular bacteria in comparison with *E. coli* is found in Fig. 8. A further analysis of these genes in obligate intracellular, facultative intracellular, host-associated, and free-living bacteria across nine major bacterial groups can be found in Atwal et al. (2021).

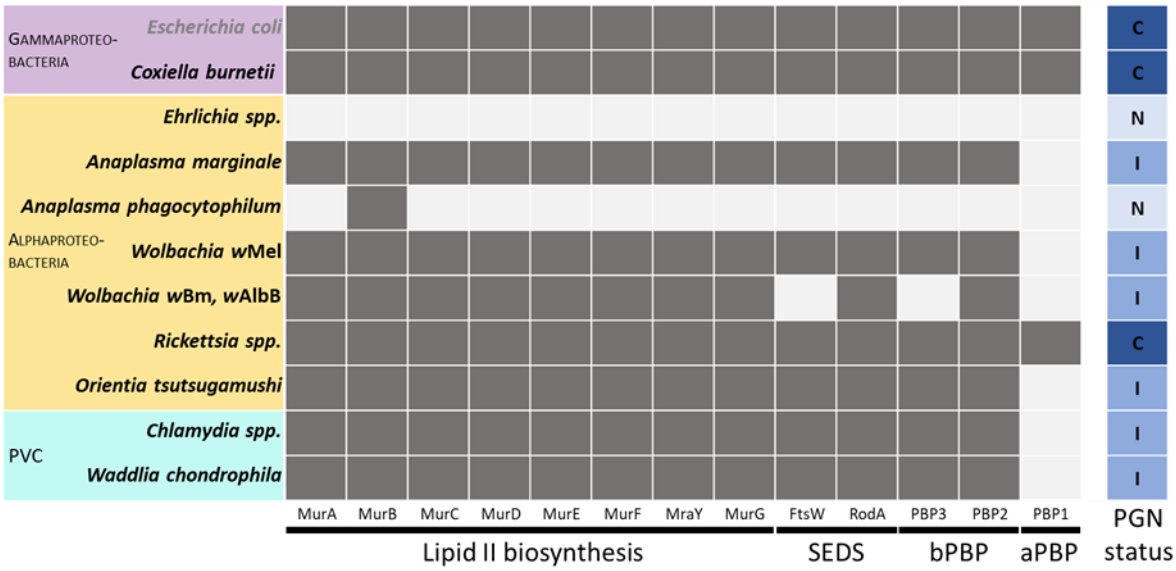

Figure 8. Peptidoglycan biosynthesis genes and status in obligate intracellular bacteria. The presence (dark grey) and absence (light grey) of key peptidoglycan (PGN) biosynthesis gene homologs in obligate intracellular bacteria is shown, with "spp." indicating multiple species within a genus. *Escherichia coli* is depicted as an example of a free-living bacterium. Bacteria are grouped into Gammaproteobacteria (purple), Alphaproteobacteria (yellow), and Planctomycetota, Verrucomicrobiota, and Chlamydiota (PVC, mint). The predicted PGN status is shown, divided into classical (C, dark blue), intermediate (I, blue), and negative (N, light blue). The figure was adapted from Otten et al. (2018) and Atwal et al. (2021).

1.3.5 Lipid II and peptidoglycan biosynthesis in *Wolbachia*

A PGN layer has not been observed in *Wolbachia*. However, genome sequencing of *w*Mel and *w*Bm revealed that the genes for PGN biosynthesis are conserved (Wu et al., 2004; Foster et al., 2005). Henrichfreise et al. (2009) found that FOS inhibits growth of *Wolbachia* strain B of *A. albopictus* (*w*AlbB), despite the apparent absence of PGN – a paradox reminiscent of the chlamydial anomaly, which I will therefore refer to as "wolbachial anomaly". They proposed that *Wolbachia* and *Chlamydia* retained lipid II biosynthesis genes for cell division. This has been confirmed for *Chlamydia*, as previously mentioned (Liechti et al., 2014; Jacquier et al., 2015b; Liechti et al., 2016) (see 1.3.4), but remains less well-characterized in *Wolbachia*.

There is only one *Wolbachia* species, however the different strains vary in their repertoire of conserved PGN biosynthesis genes (Foster et al., 2005; Dudzic et al., 2022). This has been reported for other obligate intracellular bacteria before, e.g., *Buchnera aphidicola*, where some strains are PGN-negative, while others retain a classical PGN (Atwal et al., 2021). The recently identified *Wolbachia* strain of *Howardula* (an insect-parasitic nematode) represents an extreme case: it has the smallest wolbachial genome discovered to date and has lost all genes for PGN biosynthesis and most genes for cell division, including *ftsZ* (Dudzic et al., 2022). This thesis focuses on *Wolbachia* of arthropods and filarial nematodes, which have retained key genes of the PGN biosynthesis pathway (Fig. 8).

According to the classification of Otten et al. (2018), *Wolbachia* belong to the PGN-intermediate group. They possess all genes for lipid II biosynthesis and the flippase MurJ, but lack aPBPs and monofunctional GTases. However, they do encode at least one bPBP and one SEDS protein: *w*Bm and *w*AlbB have only PBP2 and RodA, while *w*Mel additionally encodes PBP3 and FtsW. The amidase AmiD is conserved in some *Wolbachia* of arthropods but absent from *Wolbachia* of filarial nematodes. Amidases cleave the amide bond between Mur*N*Ac and L-Ala, separating the glycan strand from the peptide moiety (Vollmer et al., 2008b). The activity of AmiD from *w*Mel was confirmed *in vitro*, and a protective role against immune responses was proposed (Uehara and Park, 2007; Wilmes et al., 2017; García-del Portillo, 2020). In nematodes, both the mutualistic relationship and the lack of PGN receptor proteins might have led to the loss of amidases in their *Wolbachia* (Dziarski and Gupta, 2006; Wilmes et al., 2017). Furthermore, *Wolbachia* have conserved MetC and

GlyA, which were identified as alternative alanine racemases in *E. coli* and *Chlamydia pneumoniae*, respectively (Kang et al., 2011; De Benedetti et al., 2014), the ligase Ddl as well as the genes for *m*-DAP generation and incorporation. In contrast, only UppS/YaeS is conserved for generation of C_{55}-P. Only one PGN modification enzyme, a homolog of PBP6a of *E. coli*, a DD-CPase, is conserved (Vollmer et al., 2013; Otten et al., 2018). Like PGN-negative *Ehrlichia* and *A. phagocytophilum*, *Wolbachia* have lost the genes for LPS biosynthesis (Lin and Rikihisa, 2003; Wu et al., 2004; Foster et al., 2005).

The absence of a canonical PGN sacculus in *Wolbachia* aligns with their pleomorphic cell shape (Taylor et al., 2018; Atwal et al., 2021). However, experimental studies indicate that *Wolbachia* can synthesize lipid II. Key enzymes involved in PGN biosynthesis are expressed in *w*AlbB (Vollmer et al., 2013). *In vitro* assays with purified proteins from *w*Bm confirmed that MetC has alanine racemase activity (Vollmer et al., 2013), while MraY and MurG catalyze the synthesis of lipid I and lipid II, respectively (Henrichfreise et al., 2009). Moreover, *ex vivo* assays demonstrated lipid II biosynthesis by isolated *w*AlbB cytoplasmic membranes. Recently, D-Ala-labeling via click chemistry confirmed the presence of PGN precursors in *w*Mel (Atwal et al., 2021). In addition, *Wolbachia*-containing worm extracts activated both NOD1 and NOD2 signaling pathways in NFκB-reporter cells (Ajendra et al., 2016), and extracts from *w*Mel in cell culture also induced NOD1 (Atwal et al., 2021). NOD1 recognizes PGN fragments containing D-γ-Glu-*m*-DAP (Chamaillard et al., 2003), while NOD2 recognizes muramyl dipeptide (Mur*N*Ac-L-Ala-γ-D-Glu) (Inohara et al., 2003).

The biological relevance of lipid II for *Wolbachia* is further highlighted by the effect of cell wall biosynthesis inhibitors: apart from FOS in *w*AlbB (Henrichfreise et al., 2009), a second cell wall biosynthesis inhibitor, D-cycloserine, was found to inhibit replication of *w*Mel (Atwal et al., 2021). FOS induces an enlargement of *Wolbachia* *w*AlbB cells and redistribution of the PGN-associated lipoprotein (Vollmer et al., 2013). These enlarged cells resemble the ABs of *Chlamydia* upon beta-lactam treatment. It is not clear why beta-lactams are not effective in *Wolbachia* although they possess 2–3 PBPs (Hermans et al., 2001; Fenollar et al., 2003; Fallon, 2018; Atwal et al., 2021).

Taken together, genomic and experimental evidence strongly supports that *Wolbachia* of both arthropods and filarial nematodes can synthesize the cell wall precursor lipid II and

that lipid II plays a crucial role in wolbachial cell division. Fig. 9 provides an overview of the current knowledge on peptidoglycan biosynthesis in *Wolbachia*.

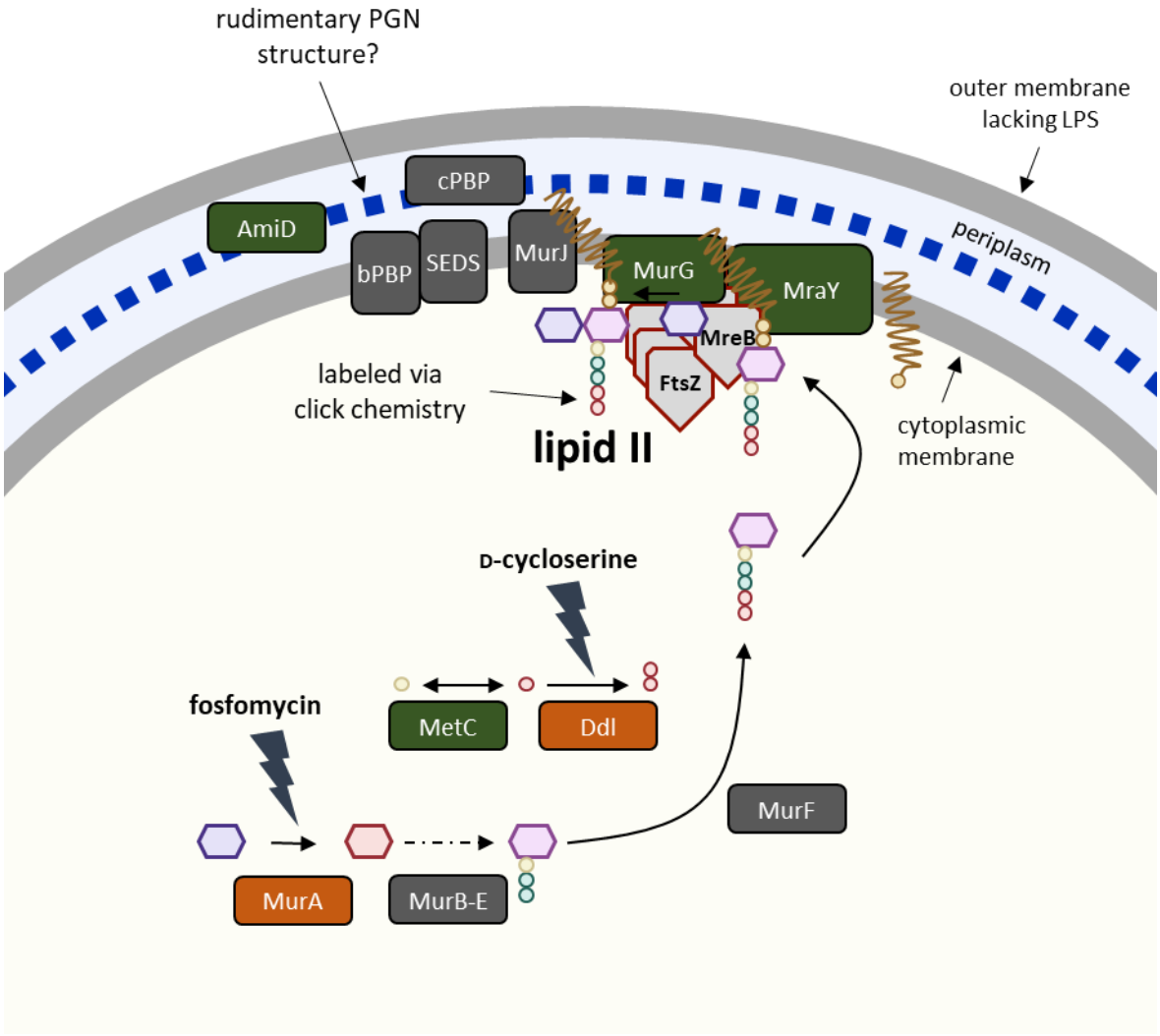

Figure 9. Peptidoglycan biosynthesis in *Wolbachia*. *Wolbachia* of arthropods and filarial nematodes have conserved an almost complete pathway for peptidoglycan (PGN) biosynthesis. Enzymes are highlighted in green if their function has been confirmed *in vitro* and in orange if they are targets of cell wall biosynthesis inhibitors active against *Wolbachia*. AmiD is conserved only in *Wolbachia* of some arthropods. From this pathway, a putative rudimentary PGN structure is predicted (blue squares in the periplasm). The outer membrane of *Wolbachia* does not contain lipopolysaccharides (LPS).

1.4 Objectives

Although *Wolbachia* are not pathogenic themselves, they play an essential role in human filarial diseases as obligate endosymbionts of filarial nematodes. Their depletion through antibiotic treatment has become a well-established strategy for combating these infections. Despite the absence of a detectable PGN sacculus, *Wolbachia* encode the complete biosynthetic pathway for the cell wall precursor lipid II. The exact role of lipid II in *Wolbachia* remains unclear, and its potential as a target for anti-*Wolbachia* intervention is not fully explored.

A better understanding of *Wolbachia* biology is crucial for developing novel anti-*Wolbachia* strategies. A key step in advancing *Wolbachia* research is the ability to study these bacteria outside their host cells. The development of an extracellular culture system would not only enable detailed investigations of *Wolbachia* biology but would also serve as a powerful tool for antibiotic screenings and future genetic manipulations. In addition, studying the cellular effects of different antibiotic classes in *Wolbachia* provides insight into its susceptibility to these antibiotics, their phenotypic effects, and potential resistance mechanisms. Enabling efficient RNA-seq of *Wolbachia* would facilitate transcriptomic analyses of antibiotic-induced cellular responses.

The main objectives of this thesis were:

1. **Establishment of a host cell-free culture of *Wolbachia***
    - Develop a culture system that enables the growth of *Wolbachia* outside of host cells.
    - Use this system as a platform for testing cell wall biosynthesis inhibitors in a host-free environment.
    - Compare the cellular effects of FOS in intra- and extracellular *Wolbachia*.

2. **Investigation of muraymycin D2 and its derivatives in *Wolbachia***

    - Evaluate the cellular effects of the cell wall biosynthesis inhibitor muraymycin D2 and its derivatives in *Wolbachia*.
    - Compare the effects of muraymycins to previously studied cell wall biosynthesis inhibitors.

3. **Analysis of resistance development against CorA in *Wolbachia***

    - Investigate whether *Wolbachia* can develop resistance against CorA, a compound in preclinical development against filarial diseases, under prolonged exposure in cell culture.

4. **Establishment of a workflow for RNA sequencing of *Wolbachia***

    - Develop an optimized protocol for RNA-seq of *Wolbachia* from infected host cells.
    - Enable future transcriptomic studies of *Wolbachia*, particularly to investigate the cellular effects of antibiotics.

2 Publications

This chapter summarizes publications (see 2.1, 2.2, 2.3) and a manuscript (see 2.4) with the contributions that were made to each. The publications can be found in the Appendix, while the manuscript is included in this chapter.

2.1 Host cell-free culture of *Wolbachia*

The study "*In vitro* extracellular replication of *Wolbachia* endobacteria" was published in Frontiers in Microbiology in 2024 (doi:10.3389/fmicb.2024.1405287). The supplementary material for this article can be found online: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1405287/full#supplementary-material.

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Contributions:

LVB: Data curation, Formal analysis (pooled data from independent experiments, statistical analysis), Funding acquisition, Investigation (analysis of insect cell lysate fractions and their combinations, replication rates in cell culture and intracellular *Wolbachia*, DNA extraction, qPCR), Methodology, Visualization, Writing – original draft, Writing – review & editing (30%, first and corresponding author)

KM: Data curation, Formal analysis, Investigation (supplementation with additional fraction 1 or cholesterol, antibiotic treatment, fluorescence microscopy, DNA extraction, qPCR), Methodology, Visualization, Writing – original draft, Writing – review & editing (first author)

JV: Data curation, Formal analysis, Funding acquisition, Investigation (testing different insect cell and *Wolbachia* numbers, cultivation with and without FBS, replication rates in cell culture and intracellular *Wolbachia*, infection assay, fluorescence microscopy, DNA

extraction, qPCR), Methodology, Visualization, Writing – original draft, Writing – review & editing (first author)

CCC: Formal analysis, Investigation (analysis of combinations of insect cell lysate fractions, DNA extraction, qPCR)

AS: Conceptualization, Writing – review & editing

AH: Conceptualization, Funding acquisition, Supervision, Writing – review & editing

KP: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing (corresponding author)

The cultivation of obligate intracellular bacteria, such as *Wolbachia*, outside of a host cell has historically been challenging due to their dependence on host cellular machinery for replication. However, developing a cell-free culture system for such bacteria is crucial for advancing genetic modification studies and other experimental manipulations. A system that supports extracellular growth could open new avenues for research into bacterial physiology, pathogenicity, and drug susceptibility (Singh et al., 2013).

Previous studies have demonstrated that *Wolbachia* can survive extracellularly for up to seven days (Rasgon et al., 2006) and can remain metabolically active in this state (Krafsur et al., 2020). However, replication in a cell-free environment has not been observed. In this study, we investigated the potential for extracellular growth of *Wolbachia* using insect cell lysate, aiming to define the conditions necessary to support cell-free bacterial replication.

Recognizing that *Wolbachia* cultivation is limited by their dependence on host cells, we tested whether insect cell lysate from C6/36 cells (derived from *A. albopictus*) could provide the essential components for bacterial replication. To this end, we adapted the protocol of Rasgon et al. (2006), omitting the final filtration step to retain insect cell lysate in the culture. The retained lysate allowed for limited replication of *Wolbachia*. We then focused on optimizing the medium composition to support extracellular growth by disrupting uninfected C6/36 insect cells and testing different concentrations. A lysate concentration equivalent to 0.95×10^6 cells/ml supported growth best. We found that a lower starting number of *Wolbachia*, as determined by 16S rRNA gene copy number, promoted higher extracellular replication rates. Replication was enabled for up to 12 days.

Subsequently, we fractionated the insect cell lysate via centrifugation to investigate which cellular components *Wolbachia* depend on for growth. The lysate was separated into three fractions: Fraction 1 (membrane fraction), Fraction 2 (cell debris and large organelles), and Fraction 3 (cytosol). Among the tested fractions, Fraction 1, which contains insect cell membranes, was the most effective in supporting extracellular *Wolbachia* growth, achieving replication levels comparable to those observed with total insect cell lysate. However, supplementation with fresh Fraction 1 on day 9 did not prolong cell-free growth. Similarly, cholesterol supplementation did not enhance growth. However, fetal bovine serum (FBS), a standard component of our cell culture medium, was identified as essential for extracellular growth. To confirm that the extracellular *Wolbachia* were not only viable and proliferating but also infective, we performed an infection assay using uninfected C6/36 insect cells. Both qPCR and fluorescence microscopy confirmed successful infection. When comparing extracellular and intracellular *Wolbachia* replication rates, we observed that extracellular growth was significantly slower. The median replication rate by day 9 was 4.2 for extracellular *Wolbachia* compared to 14.8 for intracellular bacteria. In the absence of Fraction 1, the median replication rate was 1.0, again showing that the *Wolbachia* are able to survive extracellularly but depend on factors from the insect cells for growth.

Finally, the effects of cell wall biosynthesis inhibitors, i.e., bacitracin, vancomycin, ampicillin, and FOS, on cell-free cultures were tested. Bacitracin and vancomycin are relatively large molecules, which may hinder their uptake by C6/36 insect cells. Therefore, we investigated their effects on extracellular *Wolbachia*, but neither antibiotic showed an effect. Ampicillin belongs to the beta-lactam antibiotics which were previously found to not affect intracellular *Wolbachia* either (Hermans et al., 2001; Fenollar et al., 2003; Fallon, 2018; Atwal et al., 2021) and likewise did not affect extracellular *Wolbachia*. FOS, in contrast, induces an enlarged cell phenotype in intracellular *Wolbachia* (Vollmer et al., 2013). Similarly, in our extracellular system, FOS treatment increased the cell diameter from 0.94 µm (control) to 3.36 µm. This effect was only observed when extracellular growth was enabled, indicating that FOS only acts on dividing *Wolbachia*.

Although extracellular growth of *Wolbachia* was achieved for only up to 12 days, this is a significant step toward establishing a cell-free culture system allowing for sustained growth of *Wolbachia*, and represents a valuable tool for future research.

2.2 Cellular effects of muraymycins in *Wolbachia*

The study "MraY inhibitor muraymycin D2 and derivatives induce enlarged cells in obligate intracellular *Chlamydia* and *Wolbachia* and break persistence in *Chlamydia*" was published in Antibiotics in 2024 (doi:10.3390/antibiotics13050421). The supplementary material for this article can be found online: https://www.mdpi.com/article/10.3390/antibiotics 13050421/s1.

Authors:

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Contributions:

IL: Data curation, Formal analysis, Investigation (testing muraymycins against productive and persistent *Chlamydia*, sequence alignment and structural comparison of MraY, *in vitro* analysis of MraY), Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing (first and corresponding author)

LVB: Data curation, Formal analysis (analysis of qPCR data and fluorescence microscopy images of muraymycin-treated *Wolbachia*, determination of cell diameter), Funding acquisition, Visualization, Writing – original draft, Writing – review & editing (25%, first author)

JR: Data curation, Formal analysis, Investigation (testing muraymycins against productive and persistent *Chlamydia*, *in vitro* analysis of MraY, cytotoxicity assays), Methodology, Validation, Visualization (first author)

AS: Investigation (testing muraymycins against *Wolbachia*, DNA extraction, qPCR, fluorescence microscopy), Methodology

AK: Data curation, Investigation (testing muraymycins against productive *Chlamydia*, cytotoxicity assays), Methodology, Validation, Visualization

SK: Investigation (testing muraymycins against productive *Chlamydia*), Visualization

CO: Investigation (*in vitro* analysis of MraY), Methodology, Supervision, Validation, Writing – review & editing

KM: Methodology, Writing – review & editing

SI: Resources (provision of muraymycin derivatives), Writing – review & editing

AH: Supervision, Writing – review & editing

TS: Resources, Writing – review & editing

KP: Conceptualization, Formal analysis (analysis of fluorescence microscopy images of muraymycin-treated *Wolbachia*), Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing (corresponding author)

BH: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing

Chlamydia and *Wolbachia* were previously considered cell wall-less. However, both are now classified as PGN-intermediate (Otten et al., 2018; Atwal et al., 2021) and, for *Chlamydia*, a transient septal cell wall important for cell division was discovered (Liechti et al., 2014; Packiam et al., 2015; Liechti et al., 2016) (see 1.3.4). Studying the effect of cell wall biosynthesis inhibitors can help elucidate the function of conserved cell wall enzymes and of a putative cell wall in these intracellular bacteria. In this study, we investigated effects of the MraY inhibitors muraymycin D2 and muraymycin derivatives on *Chlamydia* and *Wolbachia*. MraY is a membrane-bound enzyme that catalyzes the formation of lipid I by transferring a PGN subunit, UDP-Mur*N*Ac-pentapeptide, to C_{55}-P (Ikeda et al., 1991). We have shown that both endobacteria have retained the ability to form lipid II with recombinant purified MraY and MurG (Henrichfreise et al., 2009).

Muraymycin D2, along with all muraymycin derivatives tested, inhibited chlamydial replication. Additionally, all antibiotics except for the derivative MRH-92 induced ABs of *Chlamydia*, similar to the penicillin-induced ones. In *Wolbachia*, MRH-76 and MRH-92 reduced the relative bacterial quantity in a concentration-dependent manner, as measured by qPCR, with MRH-76 showing greater efficacy, reducing cell numbers by 68% at 32 µg/ml. Both derivatives also induced enlarged *Wolbachia* cells, similar to FOS.

To investigate whether the antibacterial activity against *Chlamydia* and *Wolbachia* in cell culture was mediated through MraY inhibition, we performed *in vitro* inhibition assays with recombinant purified MraY from *C. pneumoniae* ($MraY_{Cpn}$). Upon incubation of $MraY_{Cpn}$ with its substrates UPD-Mur*N*Ac-pentapeptide and C_{55}-P, and either muraymycin D2 or MRH-92, lipid I formation was reduced, with 0.5 µM of either antibiotic completely inhibiting the formation. To gain further insights into the enzyme's function and mode of inhibition, we performed bioinformatics analyses. Chlamydial and wolbachial MraY were compared with the well-characterized MraY from *Aquifex aeolicus*. Both 3D modeling and sequence comparison revealed that the enzyme conserved in these endosymbionts retained the amino acid residues important for binding of UPD-Mur*N*Ac-pentapeptide and muraymycins.

Although both penicillin and muraymycins induce enlarged chlamydial cells, they target different steps of cell wall biosynthesis. Thus, we investigated the effects of combined penicillin and muraymycin D2 treatment on a chlamydial infection. When treated simultaneously, inclusions containing ABs were observed, similar to penicillin treatment alone. However, when persistence was induced by penicillin and muraymycin D2 was added later, at the mid-stage of the developmental cycle, fewer inclusions were observed. Similarly, fewer inclusions were observed when muraymycin D2 was added to an active infection at the mid-stage of the developmental cycle, indicating bactericidal effects on both persisting and actively dividing *Chlamydia*.

Muraymycins represent a third class of cell wall biosynthesis inhibitors that are effective against *Wolbachia* and induce a phenotypic effect, further supporting the hypothesis that lipid II synthesis is essential for wolbachial cell division and identifying a new wolbachial target for drug development.

2.3 No resistance development against corallopyronin A in *Wolbachia*

The study “No resistance development against corallopyronin A in *Wolbachia* in C6/36 cell culture” was published in the International Journal of Antimicrobial Agents in 2024 (doi:10.1016/j.ijantimicag.2024.107344).

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Contributions:

LVB: Data curation, Formal analysis, Funding acquisition, Investigation (cell culture, long-term treatment with CorA, DNA extraction, qPCR), Visualization, Writing – original draft, Writing – review and editing (60%, first author)
CL: Data curation, Formal analysis, Investigation (cell culture, long-term treatment with CorA, DNA extraction, qPCR), Methodology
AS: Conceptualization
HN: Investigation (cell culture, MIC determination, long-term treatment with CorA, DNA extraction, qPCR)
MG: Resources (provision of CorA)
MS: Resources (provision of CorA), Supervision, Funding acquisition
GB: Formal analysis, Writing – review and editing
AH: Funding acquisition, Supervision
KP: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review and editing (corresponding author)

The antibiotic CorA is currently in preclinical development against filarial infections in humans. Just like rifampicin, it binds to the DNA-dependent RNA polymerase. However, it binds to a different site, the switch region, which prevents cross-resistance and keeps CorA active against rifampicin-resistant strains (Mukhopadhyay et al., 2008). Nevertheless, over

time, bacteria will eventually become resistant against all discovered and developed antibiotics, including CorA. The development of resistance has already been investigated for other bacteria, i.e., *S. aureus* and *N. gonorrhoeae*, for which CorA could be a treatment option as well (Balansky et al., 2022; Balthazar et al., 2024). Here we investigated the development of resistance against CorA in *Wolbachia in vitro* in our C6/36 cell culture. A high frequency of resistance to mutation would be concerning, especially in regard to the long treatment periods that are usually required for effective depletion of *Wolbachia* (Schiefer et al., 2012).

For resistance development studies, concentrations based on the minimum inhibitory concentration (MIC) are used. Therefore, the MIC of CorA against *Wolbachia* was determined first. Since *Wolbachia* are obligate intracellular bacteria, classical MIC determination via plating cannot be performed. Instead, we defined the MIC as the concentration at which the ratio of the copy numbers of *16S rRNA* (wolbachial single copy gene) to *act* (C6/36 cell actin gene) falls below 0.5 after a 9-day treatment. Using this qPCR-based MIC determination, we found the MIC to be 31.25 nM.

Wolbachia-infected insect cells were treated for 245 days with different CorA concentrations, corresponding to 0.5 × MIC, MIC, and 4 × MIC, as well as a DMSO control. The medium was changed every two days, including the addition of the corresponding antibiotic concentration. Every seven days, some of the cells were harvested for DNA extraction and qPCR analysis, while the rest were cultivated further. Over the course of the experiment, we did not observe an increase in the *16S rRNA* copy number and, thus, in the *16S rRNA*/*act* ratio. When the MIC-treated cells were challenged with 4 × MIC after 140 and 217 days, the ratio decreased to the same level as for those that were 4 × MIC-treated from the beginning.

There was no development of resistance against CorA at the selected concentrations during the observed period. Based on our *Wolbachia* number per treatment group, i.e., 2.75×10^9 *Wolbachia*, we predicted a frequency of mutation to resistance $<2.75 \times 10^{-9}$, which is lower than for *S. aureus* (1.48×10^{-8}) (Balansky et al., 2022) and might be similar to that of *N. gonorrhoeae* ($\leq 10^{-10}$) (Balthazar et al., 2024). These results support the further preclinical development of CorA against *Wolbachia* and therefore filarial infections.

2.4 RNA preparation for RNA-seq of *Wolbachia*

The study "Improved RNA preparation for RNA-seq of the intracellular bacterium *Wolbachia w*AlbB" is currently being finalized for submission.

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Contributions:

LVB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation (cell culture, RNA experiments (preservation, extraction, depletion), quality control (Experion, RT-qPCR)), Methodology, Visualization, Writing – original draft, Writing – review and editing (60%, first and corresponding author)
TAH: Data curation, Formal analysis, Writing – original draft, Writing – review and editing
AH: Funding acquisition, Supervision
KN: Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review and editing
KP: Conceptualization, Funding acquisition, Supervision, Writing – review and editing (corresponding author)

RNA-seq is used for transcriptome analysis, e.g., to investigate the cellular effects upon antibiotic treatment. However, for intracellular bacteria, such as *Wolbachia w*AlbB, RNA-seq is challenging due to the dominance of host RNA, a problem exacerbated when using non-standard, non-mammalian cell lines (Westermann et al., 2012; Kumar et al., 2016). In this study, various approaches for RNA preservation, extraction, and rRNA depletion were tested to enrich wolbachial mRNA while maintaining high RNA quality.

For RNA preservation, QIAzol (Qiagen) demonstrated superior performance compared to RNAlater (Thermo Fisher Scientific) and shock freezing, yielding higher RNA concentrations and RNA Quality Indicator (RQI) values. RNA extraction with the miRNeasy Mini Kit (Qiagen) using BCP instead of chloroform and the QIAcube automated station resulted in high

quality RNA. Several strategies for rRNA depletion were evaluated, namely the Illumina Ribo-Zero Plus Depletion Kit (Illumina), Terminator Exonuclease (Epicentre), and standard and custom riboPOOLs (siTOOLs Biotech) for Pan-Bacteria, *A. albopictus*, and *Wolbachia.* Due to very low RNA concentrations and indeterminable RQI values, Terminator Exonuclease was discontinued as an option.

In the first RNA-seq experiment, rRNA depletion was carried out using the Illumina Ribo-Zero Plus Depletion Kit. However, this kit, which is optimized for human, mouse, and rat RNA, was not effective for the non-mammalian host system, yielding 70% of insect and 22% of wolbachial rRNA reads. As a result, only 0.1% of all reads mapped to *Wolbachia*, while 88.8% mapped to *A. albopictus*. To address this, eukaryotic mRNA depletion with Dynabeads (Thermo Fisher Scientific) was introduced as an additional step. Despite the higher-than-expected loss of RNA, poly(A)-depleted RNA with high RQI and good absorption ratios was obtained, allowing subsequent combination of rRNA and mRNA depletion.

The second RNA-seq experiment compared rRNA depletion with riboPOOLs for Pan-Bacteria and *A. albopictus* with the Illumina kit. The optimal ratio of the riboPOOLs was determined as 1:4 and both methods were combined with eukaryotic mRNA depletion. While *A. albopictus* rRNA depletion was less efficient with riboPOOLs, the proportion of wolbachial reads increased slightly (0.8% vs. 0.7%). Further analysis identified most reads as mapping to *A. albopictus* pseudogenes, resembling rRNA. To address this, a custom pseudogene riboPOOL was developed by siTOOLs Biotech. Additionally, a *Wolbachia* riboPOOL was developed.

In the third RNA-seq experiment, a combination of the new pseudogene and *Wolbachia* riboPOOLs with the existing *A. albopictus* riboPOOL and eukaryotic mRNA depletion achieved unprecedented enrichment of wolbachial mRNA, with 30.2% of reads mapping to *Wolbachia*. This marks a 300-fold increase compared to our first RNA-seq experiment and the highest proportion of reads achieved in RNA-seq for intracellular *Wolbachia*.

This optimized method for RNA preparation and depletion provides a framework for transcriptomic studies of *Wolbachia*, overcoming key challenges in this field. It might be adapted for other intracellular bacteria in non-mammalian hosts.

Improved RNA preparation for RNA-seq of the intracellular bacterium *Wolbachia w*AlbB

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Keywords: *Wolbachia*, RNA-seq, transcriptome analysis, rRNA depletion, riboPOOLs, Dynabeads, mRNA depletion, endobacteria

Abstract

Despite advances in RNA-seq, investigating the transcriptome of intracellular bacteria remains challenging due to the substantial presence of host RNA. In the case of *Wolbachia* that are propagated in insect cell lines, commercially available rRNA depletion kits are often not suitable. Here, we describe a method to study the transcriptome of *Wolbachia w*AlbB in the *Aedes albopictus* cell line C6/36. To enrich the bacterial mRNA, eukaryotic mRNA was depleted by Dynabeads (Thermo Fisher Scientific). Then custom-designed riboPOOLs (siTOOLs Biotech) were used to remove both prokaryotic and eukaryotic rRNA. Compared to RNA prepared using the Illumina Ribo-Zero Plus Depletion Kit alone, additional depletion of eukaryotic mRNA increased wolbachial reads 7-fold to 0.7% of all reads. After removing

eukaryotic and prokaryotic rRNAs with custom-designed riboPOOLs, there was a 300-fold increase of reads that mapped to *Wolbachia* (30.2%). Combining customized rRNA depletion from both organisms with eukaryotic mRNA depletion was more cost-effective than simply increasing the number of sequencing reads. This method can potentially be used for the enrichment of bacterial mRNA in studies of intracellular bacteria that cannot be propagated in standard cell lines.

1 Introduction

RNA-seq has been used for almost 20 years for transcriptome analysis, offering unparalleled insights into the transcriptional landscape of cells and tissues. It is particularly useful for identifying differentially expressed genes, uncovering regulatory mechanisms, and understanding functional responses at the molecular level during processes such as development, disease, or treatment response (Wang et al., 2009; Stark et al., 2019).

Commercially available RNA-seq kits are designed to streamline the process of rRNA depletion, library preparation, and sequencing, providing high-quality results for various biological systems. Standard kits are optimized for commonly used model organisms, i.e., human, mouse, rat, or bacteria (Kumar et al., 2012; Kumar et al., 2016; Koh et al., 2023; Cantin et al., 2024). However, when working with non-standard organisms, such as insect cell lines, the suitability of these kits can vary, especially for rRNA depletion. The differences in rRNA sequences in these species from those used to design commercial rRNA depletion probes prevent effective binding of the probes, resulting in insufficient rRNA removal and rRNA contamination that can mask mRNA (Koh et al., 2023; Cantin et al., 2024). The challenge becomes even greater when studying intracellular bacteria, such as *Wolbachia,* in non-standard organisms or cell lines, as these bacteria often have small genomes and can be present in low numbers in host cells (Westermann et al., 2012; Raquin et al., 2015; Kumar et al., 2016). Consequently, transcriptome analysis of intracellular bacteria can be hampered by the presence of both bacterial and host rRNA, as well as host mRNA.

Wolbachia are Gram-negative obligate intracellular alpha-proteobacteria found in arthropods and filarial nematodes. Although they belong to the most widespread endosymbionts, with 40–52% of arthropods being infected (Zug and Hammerstein, 2012;

Weinert et al., 2015), and have medical relevance for hindering the transmission of viral diseases and treating diseases caused by filarial nematodes (Slatko et al., 2014), analysis of the transcriptome of *Wolbachia* is still difficult (Chung et al., 2020; Cantin et al., 2024).

While transcriptomics has been applied to study the effect of *Wolbachia* on their hosts, there are only a few studies on the transcriptomes of *Wolbachia* itself. There have been five studies on *Wolbachia* of the filarial nematodes *Onchocerca ochengi* (Darby et al., 2012), *Dirofilaria immitis* (Luck et al., 2014; Luck et al., 2015) and *Brugia malayi* (Grote et al., 2017; Chung et al., 2019), and five studies on *Wolbachia* of *Drosophila melanogaster* (Darby et al., 2014; Gutzwiller et al., 2015; Rainey et al., 2016; Lindsey et al., 2021) and *Aedes albopictus* (Leitner et al., 2021). Three of these investigated the effect of virus infection on *Wolbachia* (Rainey et al., 2016; Leitner et al., 2021; Lindsey et al., 2021). A recent re-analysis of the seven *Wolbachia* transcriptome data sets from non-viral studies revealed "a coordinated transcriptional response of translational proteins across diverse *Wolbachia* strains and host contexts", although fewer than 100 differentially expressed genes were identified and a general lack of global gene regulation was concluded (Chung et al., 2020). The study also found that previous *Wolbachia* transcriptomic studies might not have achieved the necessary sequencing depth for differential expression analyses of *Wolbachia*, with usually less than 10% of all reads mapping to *Wolbachia*.

Although we recently published a cell-free system that allows for the extracellular cultivation of *Wolbachia w*AlbB for 12 days (Behrmann et al., 2024), long-term culturing of *Wolbachia* is only possible *in vitro* in insect cell lines (Fallon, 2021). Several *Wolbachia*-infected insect cell lines have been established; primarily based on *A. albopictus* (e.g., C6/36 and Aa23) and *D. melanogaster* (JW18) (Fallon, 2021). This impedes their RNA analysis since insect cell rRNA depletion was less efficient with standard kits (Kumar et al., 2016). While rRNA depletion kits have been successfully developed for the depletion of common eukaryotic rRNA, i.e., human, mouse, rat, rRNA depletion kits for insect cells are limited to kits suitable for *Drosophila* (Kumar et al., 2012; Kumar et al., 2016; Koh et al., 2023; Cantin et al., 2024).

Here, we present a method based on custom-designed riboPOOLs and eukaryotic mRNA depletion, which resulted in 30% of reads mapping to the *Wolbachia w*AlbB genome. This

method should enable further studies of the wolbachial transcriptome, including upon antibiotic treatment, and might be adapted for other intracellular bacteria.

2 Methods

2.1 C6/36 insect cell culture

The *A. albopictus* C6/36 insect cell line, uninfected or infected with the *Wolbachia pipientis* supergroup B strain of *A. albopictus* (*w*AlbB), was cultured as previously described (Turner et al., 2006a; Henrichfreise et al., 2009). *Wolbachia*-infected C6/36 cells were incubated in plug-sealed 75 cm^2 culture flasks (Greiner, Kremsmünster, Austria) at 26 °C in 15 ml Leibovitz's L15 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 20% heat-inactivated FCS (PAN-Biotech, Aidenbach, Germany), 1% MEM nonessential amino acids (Thermo Fisher Scientific), 2% tryptose phosphate broth (Sigma-Aldrich, St. Louis, Missouri, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific). They were passaged every 7 days, with 3 ml of the passage added to a new flask with 12 ml fresh medium. Medium exchange (10 ml) was performed 3 days after passaging.

For RNA isolation, insect cells were harvested using a cell lifter (Corning, New York, USA), stained with trypan blue (0.4%, Thermo Fisher Scientific), and counted in a Neubauer chamber (Laboroptik, Bad Homburg, Germany). The cells were then diluted to the desired concentration and either directly centrifuged or seeded into 12-well plates (Greiner) and further incubated in a final volume of 2 ml for up to 9 days (typical duration for antibiotic assays of *Wolbachia* (Johnston et al., 2014)). Cells were harvested from the plates with a cell lifter (Sarstedt, Nümbrecht, Germany). Unless stated otherwise, cells were centrifuged at 500 g for 20 min at 4 °C (Centrifuge 5417 R, Eppendorf, Hamburg, Germany), and the supernatant carefully removed via pipetting.

2.2 RNA preservation

Different insect cell numbers were tested, i.e., 6.75×10^6, 10^5, and 10^4 cells/ml in duplicates. 200 µl cell culture of the different dilutions were harvested (160 g, 4 °C, 10 min), and various RNA preservation methods were tested: shock freezing in liquid nitrogen, RNAlater™ Stabilization Solution (Thermo Fisher Scientific), and QIAzol Lysis Reagent (Qiagen, Hilden, Germany).

For RNAlater preservation, cells were either stored in 50 µl RNAlater overnight at 4 °C, followed by supernatant removal via centrifugation (6000 g, 4 °C, 10 min), and the pellet subsequently frozen at –80 °C (RNAlater I), or the cells were directly frozen in liquid nitrogen after the addition of RNAlater (RNAlater II). In the case of QIAzol, 700 µl of the reagent was added, the pellet was vortexed until fully suspended, and then frozen in liquid nitrogen. All samples were transferred from liquid nitrogen to –80 °C for storage until RNA extraction.

In subsequent experiments, 1.8 ml of cell culture was harvested to simulate conditions suitable for drug susceptibility testing in 12-well plates, where a total volume of 2 ml is used, leaving 200 µl available for DNA extraction. In the last experiment with custom-designed riboPOOLs, 2 ml were harvested from cell culture flasks. The pellet was resuspended in 1 ml of QIAzol, an adaptation made to accommodate the increased starting material. The solution was then frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

2.3 RNA extraction

All work surfaces were cleaned with RNase AWAY (Thermo Fisher Scientific), and pipettes, tips, and reagents were exclusively used for RNA assays to prevent contamination. An FFP2 mask was worn during all RNA handling steps to minimize the risk of contamination.

Total RNA was extracted from *w*AlbB-infected C6/36 cells using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), with protocol adaptations. Samples were incubated at 37 °C in a heating block until completely thawed, ensuring that all salts were fully dissolved. After an additional 5 min incubation at room temperature, samples were mixed with 200 µl chloroform (Carl Roth, Karlsruhe, Germany) for the initial experiments or 100 µl 1-Bromo-3-chloro-2-propanol (BCP, Tokyo Chemical Industry, Tokyo, Japan). BCP was preferred over chloroform due to its reduced toxicity and the need for only half the volume (Chomczynski and Mackey, 1995). The sample-chloroform/-BCP mixture was vigorously shaken for 15 seconds, followed by a 2–3 min incubation at room temperature. Phase separation was achieved through a 15 min centrifugation at 12,000 g, 4 °C. The upper aqueous phase, containing the nucleic acids, was retained, with 350 µl applied to the Qiagen column.

We included the optional DNase I treatment to eliminate contaminating DNA. Buffer RWT was prepared with isopropanol, as recommended by the manufacturer's protocol for cases of low expected RNA yield. Column-based steps were performed manually for RNA preservation or automated using the QIAcube robotic workstation (Qiagen, Hilden, Germany) for subsequent experiments, according to the protocol. Total RNA was eluted in 30 µl of RNase-free water. 4 µl of RNA was set aside for further quality analysis, while the remaining RNA was stored at –80 °C.

2.4 Quality control

RNA concentration, A260/A280 and A260/A230 ratios were measured using the NanoVue spectrophotometer (VWR, Radnor, Pennsylvania, USA). RNA quality was assessed using the Experion Automated Electrophoresis Station (Bio-Rad, Hercules, California, USA). Based on the RNA concentrations determined by spectrophotometry, the RNA StdSens (700-7103, Bio-Rad) or HighSens (700-7105, Bio-Rad) kits were selected and used as per the manufacturer's protocol. After each depletion step, NanoVue and Experion measurements were repeated to monitor any changes in RNA concentration and quality. The "scale to global" setting was used for all virtual gels to normalize the relative intensity of the lanes in the virtual gel.

To assess the efficacy of bacterial rRNA and eukaryotic mRNA depletion, RT-qPCRs were performed to quantify the copy number of wolbachial 16S rRNA and insect cell actin transcripts. No-RT controls were included to control for DNA contamination. cDNA synthesis was carried out using LunaScript RT SuperMix Kit (New England Biolabs, Ipswich, Massachusetts, USA) with 15 ng (initial testing of poly(A) depletion), 100 ng (initial testing of riboPOOLs and Terminator exonuclease), or 5 ng (depletion with custom-designed riboPOOLs) input RNA. Subsequent 16S rRNA and actin qPCRs were performed as previously described (Makepeace et al., 2006; Henrichfreise et al., 2009; Behrmann et al., 2024).

2.5 rRNA depletion

Different methods were tested for rRNA depletion. The Illumina Ribo-Zero Plus Depletion Kit (Illumina, San Diego, California, USA) was used to deplete rRNA from total RNA or

eukaryotic mRNA-depleted RNA, following the manufacturer's protocol (see 2.7 RNA sequencing).

The rRNA depletion using riboPOOLs (siTOOLs Biotech, Planegg, Germany) was carried out on total RNA according to the manufacturer's instructions, using the maximum input of 5 µg RNA. Four different riboPOOLs were employed: Pan-Bacteria (dp-K012-26), *A. albopictus* (dp-K012-47), and custom-designed riboPOOLs for *Wolbachia* and *A. albopictus*. Initially, 1 µl of riboPOOLs was used per 20 µl reaction, as recommended by the manufacturer. Later, the riboPOOLs volume was increased to 2 µl (noted in relevant experiments), necessitating a proportional doubling of the streptavidin-coated magnetic beads. RNA clean-up was achieved through ethanol precipitation performed overnight at –20 °C, followed by washing steps the next day. When rRNA depletion with riboPOOLs was combined with eukaryotic mRNA depletion, ethanol precipitation was performed after the final depletion step.

In a third trial, the Terminator exonuclease (TER51020, Epicentre, Madison, Wisconsin, USA) was used for 2.5 µg of total RNA following the standard protocol, but without the addition of RNase inhibitor. The reaction was terminated using the riboPOOLs protocol.

2.6 Eukaryotic mRNA depletion

Eukaryotic mRNA was depleted using Dynabeads (Thermo Fisher Scientific), which have oligo $(dT)_{25}$ residues that bind to the poly(A) tail of eukaryotic mRNA. The depletion followed the manufacturer's instructions with minor adaptations. The protocol, optimized for 75 µg of total RNA in 100 µl, was adjusted to handle 20–25 µl of RNA, with the corresponding reduction in binding buffer volume to 25 µl instead of 100 µl. The supernatant, containing RNA without eukaryotic mRNA, was not discarded but retained for further processing. mRNA was eluted in 5 µl of 10 mM Tris-HCl. Ethanol precipitation was performed as described earlier before samples were analyzed via Experion and sent for RNA-seq.

2.7 RNA sequencing

To determine the most effective strategy for RNA preparation, three sequencing runs were performed using different rRNA depletion methods.

RNA samples were quality checked and quantified using the Qubit RNA HS Assay Kit and an Agilent 2100 Bioanalyzer using the RNA 6000 Pico Kit (Agilent). Library preparation was performed with the Illumina Stranded Total RNA Prep Kit, and ligation with the Illumina Ribo-Zero Plus Kit according to the manufacturer's instructions, but without the initial rRNA depletion step for already rRNA-depleted samples. In brief, 5 ng RNA was used for cDNA library construction, adapter ligation and 15 cycles of barcoding PCR using IDT for Illumina RNA UD Indexes Ligation. Obtained libraries were quantified with Qubit 1x DNA HS Assay Kit (Thermo Fisher) and the fragment distribution was checked on an Agilent 2100 Bioanalyzer using High Sensitivity DNA Kit (Agilent). Libraries were pooled equimolarly and RNA-seq was performed by the Center for Genomics and Transcriptomics (CeGaT, Tübingen, Germany; first run) and the Institute for Medical Microbiology (part of the NGS Competence Center NCCT (Tübingen, Germany); second and third run), while data management, including storage of raw data for this project were done by the Quantitative Biology Center (QBiC, Tübingen, Germany).

For the first run, libraries were sequenced as paired-end reads on a NovaSeq 6000 platform (Illumina) at a depth of 14 to 17 million reads each. For the second run, libraries were sequenced as single-end reads on a MiSeq platform at a depth of 2 to 4.5 million reads. For the third run, libraries were sequenced as single-end reads on a NovaSeq 6000 platform (Illumina) at a depth of 160 to 200 million reads.

For all three runs, sequencing statistics, including the quality per base and adapter content assessment of resulting transcriptome sequencing data were conducted with FastQC v0.11.5 (Andrews, 2015). All read mappings were performed independently against the reference strain of *Wolbachia pipientis w*AlbB (RefSeq ID: NZ_CP031221.1) and the reference strain of *A. albopictus* (RefSeq ID: GCF_006496715.1). The mappings of all samples were conducted with HISAT2 v2.1.0 (Kim et al., 2015). The first run with paired-end reads was sequenced in paired-end mode with spliced alignment enabled. For the second and third runs, spliced alignment of reads was disabled and library type was set to reverse (HISAT2 parameter --no-spliced-alignment and --rna-strandness R).

The resulting mapping files in SAM format were converted to BAM format using SAMtools v1.9 (Li et al., 2009). Mapping statistics, including percentage of mapped reads and fraction exonic region coverage, were conducted with the RNA-Seq module of QualiMap2

v2.2.2-dev (Okonechnikov et al., 2016). Gene counts for all samples were computed with featureCounts v1.6.4 (Liao et al., 2014) based on the annotation of the respective reference genome, where the selected feature type was set to transcript records (featureCounts parameter -t transcript). A quality check for rRNA was performed with a self-written script based on the absolute counts of annotated rRNAs.

3 Results

3.1 Optimization of RNA preservation

Common issues in RNA extraction are low RNA yields and degradation. To optimize RNA preservation, different methods (shock freezing, RNAlater, and QIAzol) were tested at varying insect cell concentrations (6.75×10^6, 10^5, 10^4 cells/ml). As expected, higher cell numbers resulted in increased RNA concentrations and better A260/A280 and A260/A230 ratios (data not shown). Further analysis with the Experion Station revealed that, while shock freezing produced the highest RNA concentration, it led to significant RNA degradation, with an RNA quality indicator (RQI) of 1.9 (an RQI ≥7 is recommended for RNA-seq) and multiple bands on the virtual gel (Table 3, Fig. 10, Supplementary Fig. 1). Freezing the pellet of 200 µl confluent cell culture (~10^6 cells) in QIAzol yielded the best results, providing an RQI of 10 and twice the RNA concentration compared to RNAlater samples. For all subsequent experiments, cells were preserved in QIAzol before RNA extraction.

Large rRNAs, 18S and 28S in eukaryotes and 16S and 23S in prokaryotes, are commonly assessed by gel electrophoresis to evaluate RNA integrity, with two distinct rRNA bands indicating intact RNA. However, insect cell rRNA often appears degraded, showing only one prominent band. This phenomenon is due to a “hidden break” in the 28S rRNA, which causes it to split into two fragments of similar size that co-migrate with 18S rRNA during denaturation (Gould, 1967; Ishikawa and Newburgh, 1972; Winnebeck et al., 2010).

Table 3. Experion analysis of RNA from different preservation methods at varying insect cell concentrations.

RNA preservation method	Insect cell concentration (× 6.75/ml)	Concentration (ng/µl)	RQI*
Shock freezing	10^6	118.96	1.9
	10^5	5.39	/
	10^4	1.33	/
RNAlater I	10^6	55.29	10.0
	10^5	6.47	/
	10^4	1.11	/
RNAlater II	10^6	42.63	10.0
	10^5	4.91	/
	10^4	4.77	/
QIAzol	10^6	114.63	10.0
	10^5	8.57	/
	10^4	1.61	/

*The determined RNA quality indicator (RQI) values are shown. A dash (/) indicates that RQI could not be determined due to insufficient RNA concentration. RQI values ≥7 were considered indicative of acceptable RNA quality for RNA-seq.

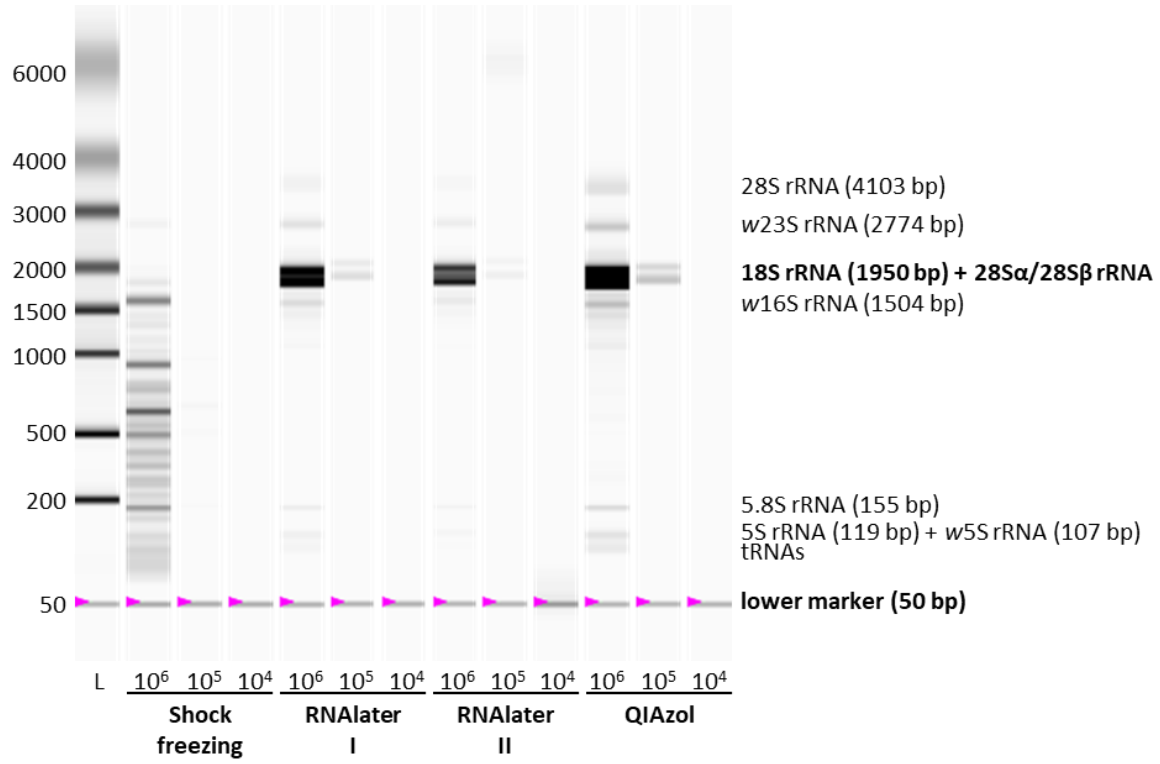

Figure 10. Virtual RNA gel after different RNA preservation methods at varying insect cell concentrations. Insect cells, at concentrations of 6.75 × 10^6, 10^5, and 10^4 cells/ml, were preserved using shock freezing, RNAlater (I: incubation overnight at 4 °C with an additional washing step before freezing, II: direct freezing), or QIAzol. RNA was extracted with the miRNeasy Mini Kit and subsequently analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol on the Experion Automated Electrophoresis Station. The resulting virtual gel is shown, with insect and wolbachial rRNA as well as tRNA bands labeled.

3.2 Enrichment for wolbachial reads via rRNA and eukaryotic mRNA depletion

Because no commercial rRNA depletion kit is available for RNA-seq of *Wolbachia*, we tested various rRNA depletion methods with and without eukaryotic mRNA depletion.

In our first trial, total RNA with excellent quality (RQI of 10, Supplementary Fig. 2) was subjected to rRNA depletion with the Illumina Ribo-Zero Plus Depletion Kit. Only 0.1% wolbachial reads were achieved, with inefficient rRNA depletion for both *A. albopictus* (70.3% rRNA reads) and *Wolbachia* (22.0% rRNA reads). Due to the overwhelming abundance of eukaryotic mRNA compared to bacterial mRNA, we opted for using Dynabeads for poly(A) depletion rather than eukaryotic mRNA enrichment, as previously described (Kumar et al., 2016). We first evaluated the impact of poly(A) depletion on RNA integrity and quantity. We recovered 56% of poly(A)-depleted RNA from total RNA, with

excellent A260/A280 and A260/A230 ratios (Table 4). Due to high salt concentrations and EDTA in the Dynabeads binding buffer, direct analysis via Experion was not possible (Fig. 11A+B, Supplementary Fig. 3). After diluting the sample, Experion analysis was possible and an RQI of 10 was determined (Fig. 11C, Table 4). cDNA synthesis followed by actin qPCR confirmed a 77% reduction in actin transcripts. 16S rRNA transcripts were found to increase by 150% (data not shown), likely attributable to the effective reduction of eukaryotic transcripts, thus increasing the relative proportion of wolbachial transcripts.

Analysis of the mRNA fraction showed that capturing of the eukaryotic mRNA was successful, yielding 0.8% mRNA from total RNA, with 10.1% rRNA contamination (as measured on the Experion Station). Actin transcripts were enriched 300-fold in mRNA compared to total RNA.

Table 4. RNA quality analysis after poly(A) depletion.

	Sample	c (ng/µl)	% of total RNA	RQI	A260/A280[a]	A260/A230[a]
1	total RNA[b]	462.0	100	10	1.99	2.39
	poly(A)-depleted[c]	157.5	76.71	10	2.04	2.19
	eukaryotic mRNA[d]	15.5	0.84	-	2.07	0.98
2	total RNA	468.0	100	10	2.07	2.43
	poly(A)-depleted	129.0	55.13	10	2.04	2.31
	eukaryotic mRNA	22.9	0.98	-	2.07	1.36
3	total RNA	1173.2	100	10	2.07	2.35
	poly(A)-depleted	190.7	36.56	10	2.04	2.17
	eukaryotic mRNA	21.9	0.47	-	2.13	1.47

[a]The A260/A280 and A260/A230 ratios were determined with a NanoVue spectrophotometer.
[b]Total RNA was extracted from 0.5×10^7 cells/ml cultured for 6 days (samples 1, 2) or 9 days (sample 3) in 12-well plates with the miRNeasy Mini Kit. Poly(A) depletion was performed and the resulting poly(A)-depleted RNA and eukaryotic mRNA rescued. Total RNA was analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol on the Experion Automated Electrophoresis Station. The total RNA of samples 1 and 3 was diluted 1:1 before measuring.
[c]Poly(A)-depleted RNA (1:100 diluted) was analyzed with the HighSens Kit using the eukaryotic total RNA protocol on the Experion Automated Electrophoresis Station.
[d]Eukaryotic mRNA was analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol on the Experion Automated Electrophoresis Station. A hyphen (-) indicates that RQI was not determined due to selection of the mRNA protocol.

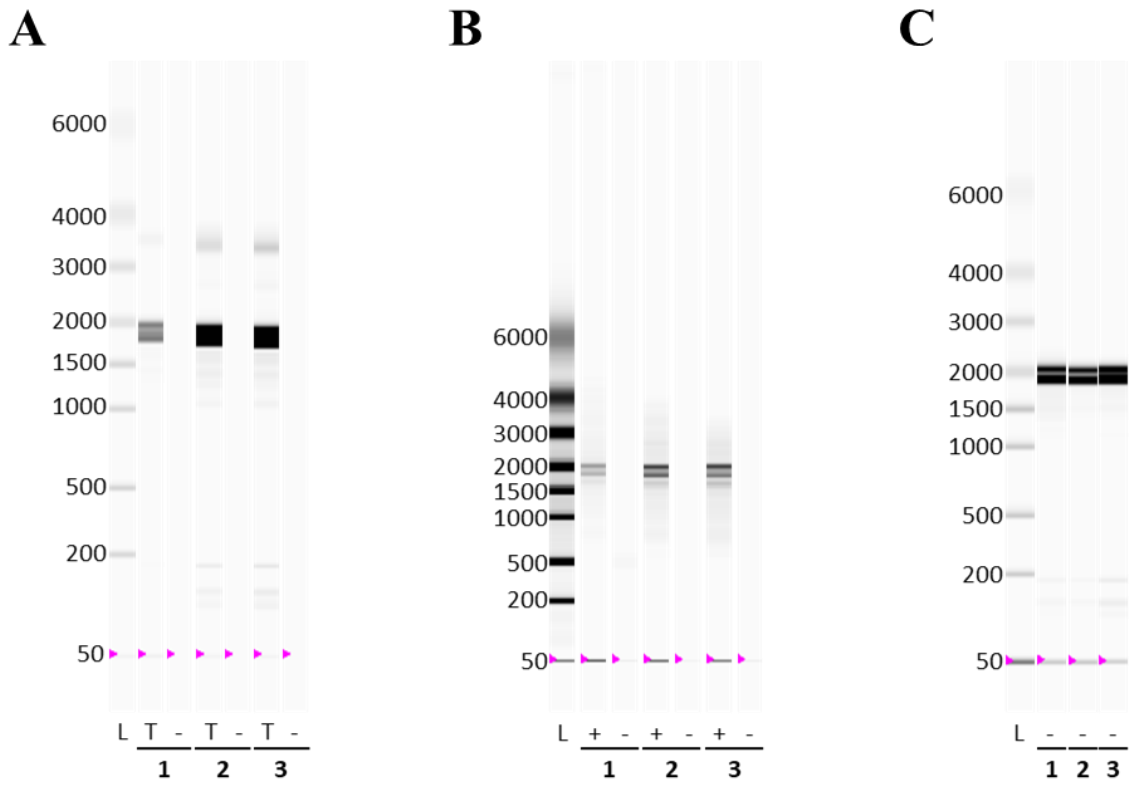

Figure 11. Virtual RNA gels after poly(A) depletion. Total RNA was extracted from 0.5 × 10^7 cells/ml cultured for 6 days (samples 1, 2) or 9 days (sample 3) in 12-well plates with the miRNeasy Mini Kit. Poly(A) depletion was performed and the resulting poly(A)-depleted RNA and eukaryotic mRNA rescued. The different types of RNA were analyzed on the Experion Automated Electrophoresis Station and the virtual gels are shown. (**A**) Total RNA (T) and poly(A)-depleted RNA (-) were analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol. The total RNA of samples 1 and 3 was diluted 1:1 before measuring. (**B**) Eukaryotic mRNA (+) and poly(A)-depleted RNA (-) were analyzed with the Experion StdSens Kit using the mRNA protocol. (**C**) Poly(A)-depleted RNA (-) was diluted 1:100 and analyzed with the Experion HighSens Kit using the eukaryotic total RNA protocol.

Next, we tested riboPOOLs that were designed for bacterial and *A. albopictus* rRNA depletion. Different ratios of the Pan-bacteria and *A. albopictus* riboPOOLs were tested and their efficiency assessed via 16S rRNA RT-qPCR (Fig. 12A) and Experion analysis (Fig. 12B, Supplementary Fig. 4). A 1:4 ratio of Pan-bacteria to *A. albopictus* riboPOOL was found to lead to the best depletion. As expected, a 1:1 ratio showed stronger depletion of the wolbachial 16S rRNA (98%), while 1:4 and 1:9 ratios led to more depletion of the insect cell rRNA as visible from weaker bands on the virtual gel, with RNA concentrations equaling 9% and 10% of total RNA, respectively. Due to substantial 16S rRNA detected in the 1:9 samples via RT-qPCR (only 32% reduction) and on the virtual gel, the 1:4 ratio was chosen.

Integration of the 5S, 18S, and 28S rRNA peaks and normalization to RNA concentration determined an rRNA content of 61% in samples with a 1:4 ratio.

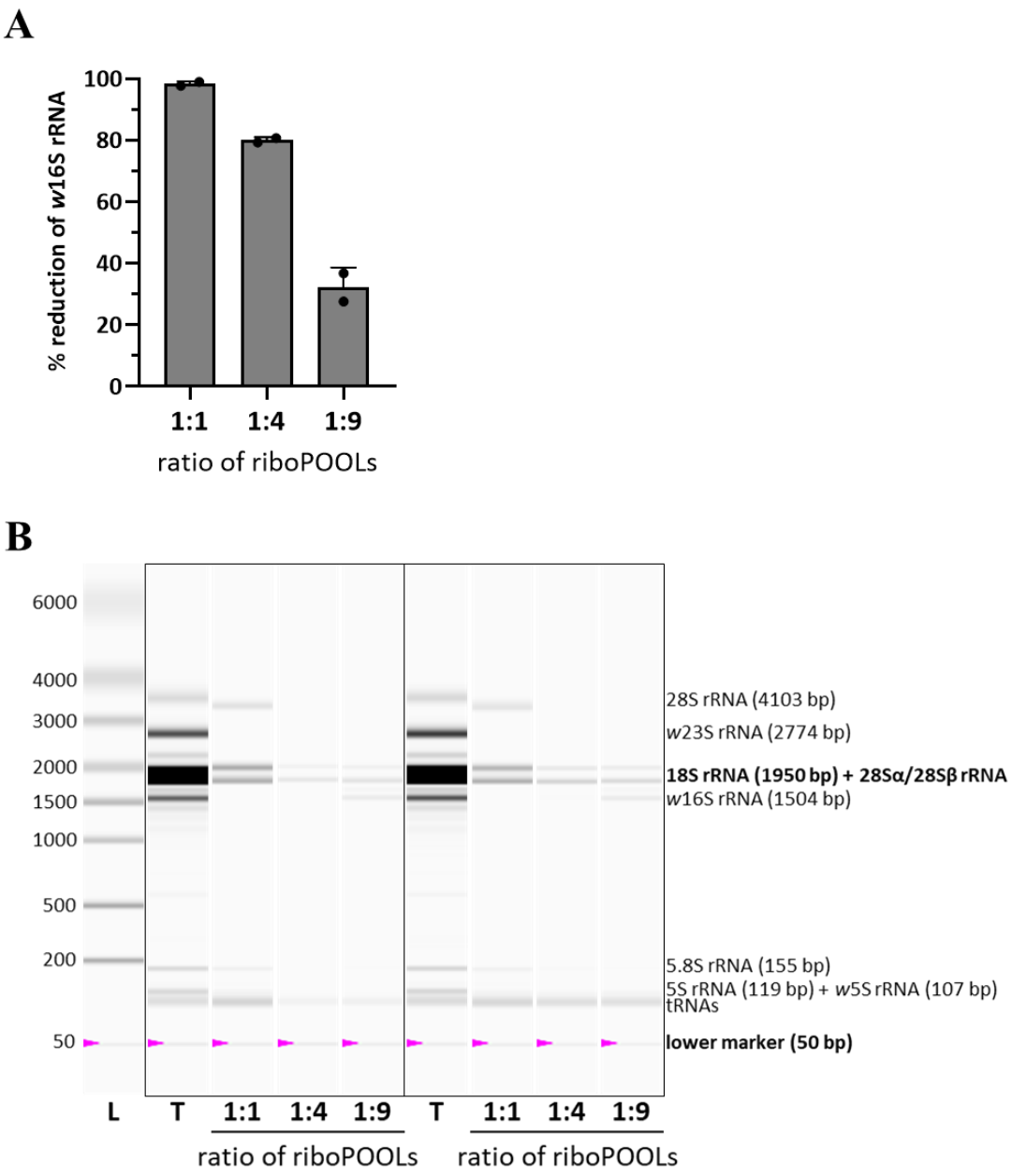

Figure 12. Ratio-dependent depletion of wolbachial and insect cell rRNA by riboPOOLs. Total RNA was extracted with the miRNeasy Mini Kit in technical duplicates and subsequently treated with different ratios of Pan-bacteria to *Aedes albopictus* riboPOOL (1:1, 1:4, 1:9). (**A**) Wolbachial 16S rRNA (*w*16S rRNA) copies were measured via RT-qPCR. The reduction was calculated compared to the total RNA. The mean ± SD is shown. (**B**) Total RNA (T) and RNA after treatment with different ratios of riboPOOLs were analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol on the Experion Automated Electrophoresis Station. The resulting virtual gel is shown, with insect and wolbachial rRNA as well as tRNA bands labeled.

rRNA depletion with Terminator exonuclease was tested as well, however RNA concentrations were so low that we did not continue with this approach.

As poly(A) depletion was successful, we then combined it with rRNA depletion either with the Ribo-Zero Plus Depletion Kit or with riboPOOLs. We performed biological duplicates with technical duplicates for each condition. The electropherograms show that rRNA depletion reduced the rRNA peaks (Supplementary Fig. 5). The samples of the second biological replicate (virtual gels in Fig. 13) were then RNA-sequenced.

For the combination with Ribo-Zero Plus, the eukaryotic mRNA depletion led to increased rRNA reads (83.9% for *A. albopictus*, 37.9% for *Wolbachia*). Still, the percentage of reads mapping to *A. albopictus* was successfully decreased and the wolbachial reads increased to 0.7%, representing a 7-fold increase.

For the combination with riboPOOLs, despite the use of an *A. albopictus*-specific riboPOOL, rRNA reads constituted 92.2% for *A. albopictus*, higher than for Ribo-Zero Plus. The majority of the reads mapped to genes considered pseudogenes of *A. albopictus* in the genome annotation. Yet, the free text annotation description stated that the genes are rRNA genes. To investigate the similarity of these sequences to explicitly labeled rRNA genes, we created a multiple sequence alignment using MUSCLE (Madeira et al., 2024). The pseudogenes showed high similarity to labeled rRNA genes. Therefore, they were suspected to be rRNA genes and included into the estimation of the rRNA content. Nonetheless, wolbachial rRNA reads were reduced and with 0.8%, a slightly higher amount of wolbachial reads was achieved. This experiment confirmed the effectiveness of poly(A) depletion.

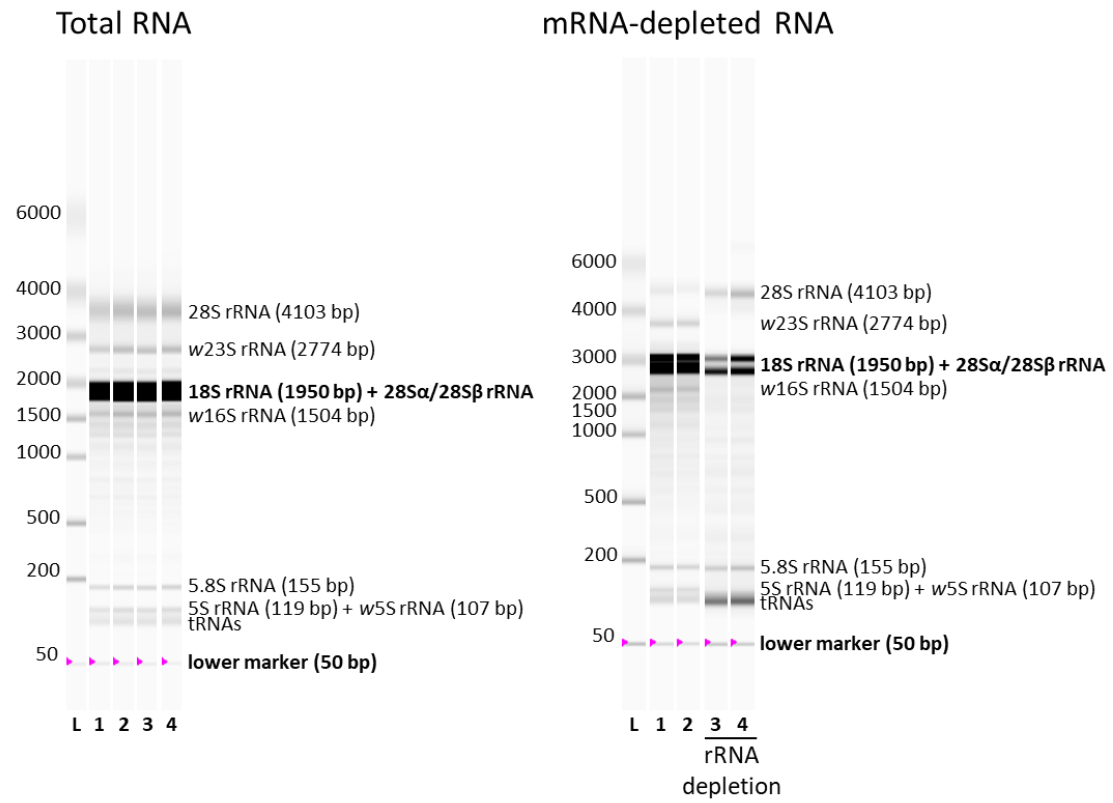

Figure 13. Virtual gels of poly(A)-depleted and rRNA- and poly(A)-depleted RNA. Total RNA was extracted with the miRNeasy Mini Kit in 4 technical replicates. Poly(A) depletion was performed with Dynabeads. rRNA depletion was performed using a 1:4 ratio of Pan-Bacteria to *Aedes albopictus* riboPOOL. Afterwards, poly(A) depletion was performed with Dynabeads. All RNA types were subsequently analyzed on the Experion Automated Electrophoresis Station. Total RNA was analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol while the poly(A)-depleted RNA and rRNA- and poly(A)-depleted RNA were analyzed with the Experion HighSens Kit using the eukaryotic total RNA protocol. The poly(A)-depleted RNA was diluted 1:500 and the rRNA- and poly(A)-depleted RNA was diluted 1:10. The resulting virtual gels are shown, with insect and wolbachial rRNA as well as tRNA bands labeled. The depleted samples were sent for RNA-seq.

3.3 Custom-designed riboPOOLs

Closer examination revealed that the pseudogenes suspected to encode rRNA form two distinct clusters in the phylogenetic tree. To reduce the number of genes, we filtered for sequences with less than 95% identity, which resulted in 12 distinct sequences. siTOOLs Biotech provided information on sequences targeted by their *A. albopictus* riboPOOL without mismatch, revealing that eight of the twelve sequences were already targeted. We confirmed that no reads mapped to these eight sequences, indicating effective depletion.

The four remaining, untargeted sequences included two pseudogenes for the large subunit, one pseudogene for the small subunit, and one gene for 5.8S rRNA. Analysis of the RNA-seq reads revealed that these untargeted sequences accounted for >85% of all *A. albopictus* reads. We sent the untargeted sequences to siTOOLs Biotech, who then developed a pseudogene riboPOOL.

Additionally, a custom-designed riboPOOL was created for *Wolbachia* to improve rRNA depletion. The new riboPOOL efficiently depleted 16S rRNA in a test extraction, as confirmed by the absence of the 16S rRNA peak in the electropherograms (Supplementary Fig. 6). For a combination with the original *A. albopictus* riboPOOL, the total amount of riboPOOLs was increased from 1 to 2 µl. Since this led to smaller rRNA peaks in the electropherograms, the higher amount was used for the following experiments. However, we noted an increase for the putative peak for tRNAs.

In the next attempt, the *Wolbachia* riboPOOL was combined with both *A. albopictus* riboPOOLs. The ratio of 1:4 was adapted to 3:8:4 for *Wolbachia*, *A. albopictus* and pseudogene riboPOOLs. The *Wolbachia* to total *A. albopictus* riboPOOL ratio was therefore still 1:4. rRNA depletion with these custom-designed riboPOOLs was combined with poly(A) depletion. RT-qPCR results were promising, with 96% 16S rRNA and 92% actin depletion (data not shown), indicating effective wolbachial rRNA and eukaryotic mRNA depletion. The virtual gel showed no (samples 1–4) or weak (sample 5) bands for rRNA post-depletion (Fig. 14A). The electropherograms of samples 3 and 4 are shown pre- and post-depletion (Fig. 14B). The peaks for 5S, 5.8S, 16S, 18S, 23S, and 28S rRNA were completely absent after depletion. However, low molecular weight RNAs, likely tRNAs, were still present after depletion. It is important to note that the intensity of the bands cannot be compared between gels. The “scale to global” setting was used which means that the fluorescence value of the tallest peak from all electropherograms in one run is used to set the scale of the y-axis for all samples and thus the relative intensity of the lanes in the virtual gel. The intensity of the first peak was still increased for the depleted samples as can be seen from the electropherograms. This can again be due to the shift in composition of RNA-species as described earlier.

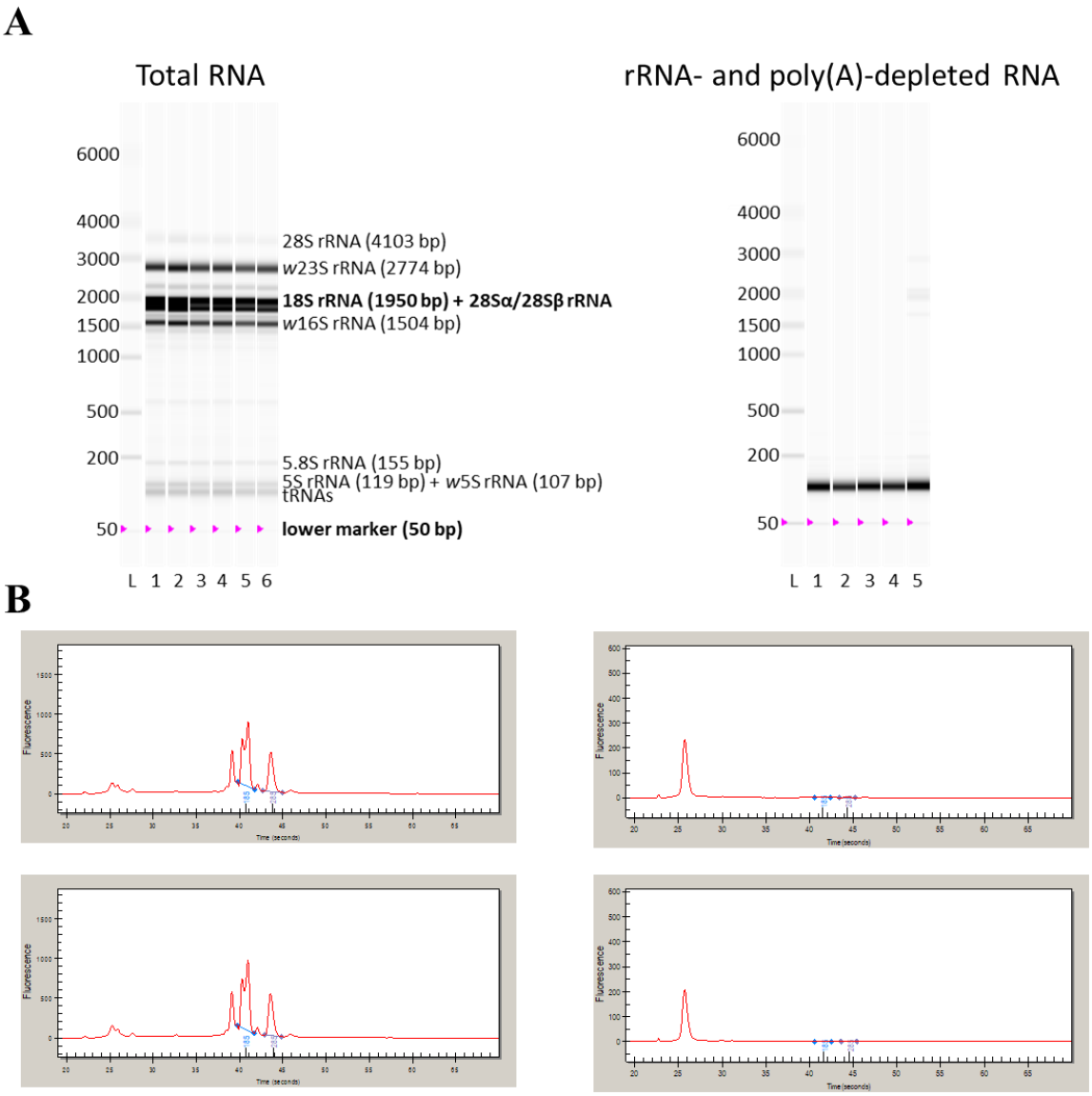

Figure 14. Poly(A) depletion and rRNA depletion with custom-designed riboPOOLs. Total RNA was extracted with the miRNeasy Mini Kit in technical replicates and rRNA depletion with riboPOOLs and subsequent poly(A) depletion were performed. (**A**) Total RNA (six replicates) was analyzed with the Experion StdSens Kit, rRNA- and poly(A)-depleted RNA (five replicates) with the Experion HighSens Kit, both using the eukaryotic total RNA protocol on the Experion Automated Electrophoresis Station. The resulting virtual gels are shown, with insect and wolbachial rRNA as well as tRNA bands labeled. (**B**) Electropherograms before (left, total RNA) and after depletion (right, rRNA- and poly(A)-depleted RNA) for the two replicates (3, 4) that were RNA-sequenced. Note the different scales of the y-axis.

This sequencing led to 48.2% of *A. albopictus* reads, of which 55.2% were rRNA. Still, the wolbachial reads were increased to 30.2%, with 32.0% rRNA. The median coverage was 166.5X, with 75.3% of the genome covered at ≥30X, ensuring sufficient depth for transcriptome analysis of *Wolbachia*. Table 5 summarizes the different RNA-seq runs in this study.

Table 5. Overview of RNA-seq mapped reads after the different RNA preparation methods in this study.

		rRNA depletion[a]	rRNA + poly(A) depletion[b]		Custom-designed rRNA + poly(A) depletion[c]
rRNA depletion		Ribo-Zero Plus	Ribo-Zero Plus	riboPOOLs	custom-designed riboPOOLs
poly(A) depletion		-	Dynabeads	Dynabeads	Dynabeads
Mapped to *A. albopictus*	% of all reads	**88.8** (88.0–89.6)	**62.1** (61.5–62.6)	**79.5** (78.3–80.7)	**48.2** (48.0–48.4)
	% rRNA genes	**70.3** (68.8–71.3)	**83.9** (83.3–84.6)	**92.2** (91.8–92.7)	**55.2** (52.9–58.7)
Mapped to *w*AlbB	% of all reads	**0.1** (0.1–0.1)	**0.7** (0.7–0.7)	**0.8** (0.8–0.8)	**30.2** (29.9–30.4)
	% rRNA genes	**22.0** (18.8–24.5)	**37.9** (33.9–41.9)	**17.3** (11.2–23.4)	**32.0** (27.0–37.0)

[a]Run was performed in triplicate on a NovaSeq 6000 platform (14–17 million reads).
[b]Run was performed in duplicate for each combination on a MiSeq platform (21–45 thousand reads).
[c]Run was performed in duplicate on a NovaSeq 6000 platform (160–200 million reads).

4 Discussion

To ensure high RNA integrity and concentration, we optimized the RNA extraction and preservation conditions, with ~10^6 insect cells as starting material together with QIAzol leading to the best results (Table 3, Fig. 10, Supplementary Fig. 1). Substituting chloroform with BCP proved advantageous, as it is less toxic, requires only half the volume, and results in a more distinct phase separation (Chomczynski and Mackey, 1995).

Quality control is essential to assess RNA integrity before proceeding with sequencing. We performed cDNA synthesis followed by qPCR targeting 16S rRNA to control for bacterial rRNA depletion and, when poly(A) depletion was included, targeting actin to control for eukaryotic mRNA depletion. For adapting our method, it might be cost-effective to include a eukaryotic rRNA RT-qPCR to the workflow as well, as others have done (Fauver et al., 2019; Goraichuk et al., 2024). An 18S rRNA RT-qPCR, already established for several mosquito species (Hoffmann et al., 2004), offers a way to control for eukaryotic rRNA depletion.

The Experion Station (Bio-Rad) was used to assess RNA quantity and quality, but it has since been discontinued, as has the formerly widely used 2100 Bioanalyzer (Agilent). Alternatives to these chip-based methods, which calculate RNA integrity numbers (RQI for Bio-Rad; RIN for Agilent), are traditional gel-based analysis of RNA integrity or other platforms for the capillary electrophoresis-based analysis, e.g., from Agilent (4150 TapeStation, 4200 TapeStation, Fragment Analyzer) or Qiagen (QIAxcel Connect). Thermo Fisher Scientific offers a method for RNA integrity determination based on fluorescent dyes (Qubit RNA IQ Assay for Qubit 4 or Qubit Flex).

We succeeded in achieving high quality RNA by using at least 10^6 cells as starting material, QIAzol, the miRNeasy Mini kit with BCP, and the QIAcube automated station for RNA extraction. Under these conditions, RNA concentrations were above 500 ng/µl, with A260/A280 1.9–2.0 and an RQI of 10.

Our sequencing experiments employed various rRNA depletion strategies, i.e., the Illumina Ribo-Zero Plus Depletion Kit, standard riboPOOLs (siTOOLs Biotech), and custom-designed riboPOOLs (siTOOLs Biotech). All of these methods were combined with poly(A) depletion with Dynabeads (Thermo Fisher Scientific), although Ribo-Zero Plus was tested without it in the first experiment.

The first RNA-seq (14–17 million total reads) led to only 0.1% reads mapped to *Wolbachia*. After performing an additional eukaryotic mRNA depletion with Dynabeads, we had 0.7% mapped to *Wolbachia* and 0.8% when we changed from the standard Ribo-Zero Plus rRNA depletion to specific riboPOOLs for Pan-Bacteria and *A. albopictus*. Although rRNA reads were increased for the riboPOOLs samples, wolbachial reads were increased, confirming the efficiency of poly(A) depletion.

Pseudogenes of *A. albopictus* were identified as rRNA genes not targeted by these riboPOOLs. We therefore recommend checking for unannotated rRNA genes should one have trouble with rRNA depletion even after using specifically designed probes. Additionally, 5.8S rRNA was not efficiently removed. The design of a new riboPOOL for these genes as well as a *Wolbachia* riboPOOL and combining all three riboPOOLs led to 30.2% of reads mapping to *Wolbachia* in the third RNA-seq (160–200 million total reads). Despite the almost complete removal of the peaks for 18S and 28S rRNA in the Experion electropherograms for the samples depleted with custom-designed riboPOOLs, relatively high percentages of rRNA were detected in RNA-seq (Table 5). We observed bands for low-molecular weight RNAs on the virtual gel (Fig. 14A) as well as a prominent peak in the electropherograms (Fig. 14B). After rRNA depletion, low-molecular weight RNAs that were previously undetected in total RNA can become detectable due to the reduced dominance of rRNA, increasing the relative abundance of tRNAs and improving detection sensitivity. Additionally, the removal of rRNA allows RNA-binding dyes to bind more effectively to tRNAs, enhancing their fluorescence signals during analysis. Wahl et al. (2022) observed the same phenomenon when using riboPOOLs for bacterial rRNA depletion. Many of the remaining reads mapped to a small nucleolar RNA (snoRNA) and a small nucleus RNA (snRNA). Still, it remains unclear why 55.2% rRNA reads for *A. albopictus* and 32.0% rRNA reads for *Wolbachia*, respectively, remained.

The custom-designed riboPOOLs led to a substantial increase in wolbachial reads, demonstrating their effectiveness in enriching bacterial RNA. Given this success, it should be tested whether Dynabeads may no longer be necessary. RNA is lost during poly(A) depletion (Table 4) and Dynabeads can potentially contaminate or inhibit downstream assays (RT-qPCRs, RNA-seq) and may also bind to prokaryotic RNA containing adenine-rich regions. Omitting poly(A) depletion in future experiments would allow for dual RNA-seq, enabling the simultaneous sequencing of both bacterial and eukaryotic transcripts.

Furthermore, while the increased coverage of the wolbachial transcriptome could be attributed to improved RNA enrichment, the overall number of reads was also 11-fold higher for the final samples that were rRNA-depleted with custom-designed riboPOOLs compared to the samples depleted with Ribo-Zero Plus. As a result, the general increase in read count likely contributed to the higher coverage and depth. Given the higher coverage,

the number of reads can be reduced to lower RNA-seq costs. For bacteria, 5–10 million non-rRNA reads were found to yield sufficient sequencing depth, with 2–3 million being enough for biological replicates (Haas et al., 2012). To achieve 5 million non-rRNA reads with our method, 24 million reads would be needed, allowing for a 5- to 10-fold reduction in total RNA-seq reads.

Our custom-designed *Wolbachia w*AlbB riboPOOL has been added to the commercial repertoire of siTOOLs Biotech (dp-K012-86), the pseudogene riboPOOL is available on request. While the use of custom-designed probes offers significant advantages in enriching specific RNA, it also comes with limitations. Custom probes are tailored for specific species and are not universally applicable. For instance, Koh et al. (2023) found that when using probes designed for *A. aegypti* on other species like *A. albopictus* and Culicine and Anopheline mosquitoes, 46–94% of reads post-depletion were still ribosomal. Similarly, the two *Aedes albopictus* riboPOOLs might not be suitable for other mosquito species. Wolbachial rRNA might be more conserved and future studies will show whether our *Wolbachia w*AlbB riboPOOL is also effective for other *Wolbachia* subspecies.

To our knowledge, our study represents the first RNA-seq study of *Wolbachia w*AlbB in an *A. albopictus* cell line. To contextualize our work, we have compiled a table summarizing the RNA-seq studies of *Wolbachia* that we found, offering a comparison of RNA depletion methods and percentage of wolbachial reads (Table 6). One study investigating the effect of virus infection on *Wolbachia* did not give the percentage of wolbachial reads from all reads and was therefore excluded (Lindsey et al., 2021).

While the percentage of mapped reads is an important metric, it must be interpreted with caution. Without knowing how many of these reads correspond to rRNA genes, this value alone may not provide a comprehensive understanding of genome coverage. Luck et al. (2014), e.g., mentioned that of wolbachial reads, >75% were rRNA reads. However, the amount of wolbachial rRNA reads was not given in all of these studies.

Table 6. Overview about RNA-seq studies of *Wolbachia* in chronological order.

Wolbachia strain	wolbachial reads (%)[a]	rRNA depletion method	poly(A) depletion	study
***w*Oo**	5	no	no	Darby et al. (2012)
***w*MelPop-CLA**	6 (M)	Terminator exonuclease	no	Darby et al. (2014)
***w*Di**	0.7 (M)	no	no	Luck et al. (2014)
***w*MelPop**	3	no	no	Woolfit et al. (2015)
***w*MelCS**	1			
***w*MelPop-CLA**	18			
***w*MelPop-CLA**	8	RiboMinus Eukaryote Kit + MicrobExpress bacterial mRNA Enrichment Kit		
purified *w*MelPop-CLA	83			
***w*Mel**	1.6 (Mdn)	RiboMinus Eukaryote Kit	no	Gutzwiller et al. (2015)
***w*Di**	9 (M)	no	no	Luck et al. (2015)
***w*Mel**	4.9 (M)	Ribo-Zero Magnetic Gold Kit (Human/Mouse/Rat)*	no	Rainey et al. (2016)
***w*Ana**	1.8	no	no	Kumar et al. (2016)
	1	Ribo-Zero Kits (Human/Mouse/Rat + Bacteria)*	yes	
***w*Bm**	1.5 (M)	no	no	Luck et al. (2017)
	1.4 (M)			
	6.3 (M)	Cappable-Seq[#]		
***w*Bm**	3.0 (M)	Terminator exonuclease	no	Grote et al. (2017)
***w*Bm**	0.7 (M)	Ribo-Zero Kits (Human/Mouse/Rat + Bacteria)*	yes	Chung et al. (2019)
	2.5 (M)	Agilent Sure Select Kit[#] (Mosquito)	no	
	24.0 (M)	Agilent Sure Select Kit[#] (Gerbil)		
***w*AlbB**	29.7 (M)	Ribo-Zero Magnetic Gold Kit (Human/Mouse/Rat)*	no	Leitner et al. (2021)

***w*AlbB**	2	VAHTS Total RNA-seq (Human/Mouse/Rat) Library Prep Kit	no	Hussain et al. (2023)
***w*Bm**	1	custom filarial nematode probes	no	Cantin et al. (2024)
***w*AlbB**	0.1 (M)	Ribo-Zero Plus Kit	no	this study
	0.7 (M)		yes	
	0.8 (M)	riboPOOLs (Pan-Bacteria + *Aedes albopictus*)		
	30.2 (M)	custom riboPOOLs (*w*AlbB + *Aedes albopictus*)		

[a]If known, it is stated whether the mean (M) or median (Mdn) are given. The wolbachial reads were given or calculated based on data deposited in the Sequence Read Archive (Darby et al., 2014), data in the manuscript (Grote et al., 2017; Luck et al., 2017; Chung et al., 2019; Leitner et al., 2021), or data provided by the authors (Rainey et al., 2016).
*Discontinued kits are marked with an asterisk.
[#]Indirect rRNA depletion via enriching for mRNA.

The study of intracellular bacteria in non-mammalian hosts is hampered by the lack of commercially available rRNA depletion kits. While some kits have been shown to work for non-mammalian rRNA depletion or were specifically designed for this purpose, availability has been a recurring issue. For example, the MICROBEnrich insect/*C. elegans* module (Ambion) is no longer available (Kumar et al., 2012). However, Thermo Fisher Scientific still offers a MICROBEnrich Kit for depletion of mammalian rRNA and can provide sequences from the discontinued modules for ordering oligos independently (personal communication: Thermo Fisher Scientific Tech Support). This approach has been successfully applied for insect rRNA depletion (Scully et al., 2013).

After the discontinuation of the insect module, Kumar et al. (2012) reported that the Ribo-Zero Kit (Human/Mouse/Rat) (Epicentre) efficiently removes over 98% of insect rRNA. This kit, later marketed by Illumina, was used alongside the Ribo-Zero Kit (Bacteria) in two RNA-seq studies of *Wolbachia* (Kumar et al., 2016; Chung et al., 2019). However, both kits were discontinued in 2018, further constraining available options. Similarly, the Ribo-Zero Magnetic Gold Kit (Human/Mouse/Rat) (Illumina), which also targets mitochondrial rRNA and was used in two RNA-seq studies of *Wolbachia*, was also discontinued (Rainey et al., 2016; Leitner et al., 2021). Using this kit, Leitner et al. (2021) achieved 29.7% reads for

*w*AlbB in an *A. aegypti* cell line, whereas Rainey et al. (2016) achieved 4.9% reads for *w*Mel in a *D. melanogaster* cell line (Rainey et al., 2016; Leitner et al., 2021). The differences in the percentage of wolbachial reads between these two studies could be due to variations in multiplicity of infection and rRNA depletion efficiency across host cells. Notably, neither study reported the percentage of rRNA reads.

We have tested the replacement product, the Ribo-Zero Plus Depletion Kit (Illumina) for human, mouse, rat, and bacteria, and found that the depletion of insect rRNA was not efficient (Table 5). Bacterial rRNA depletion was more efficient and led to a reduction to 22%. This approach yielded only 0.1% wolbachial reads. Darby et al. (2014) and Grote et al. (2017) used Terminator exonuclease (Epicentre) for rRNA depletion and achieved a mean of 6% and 3% wolbachial reads, respectively. However, they did not state the remaining rRNA content. We also tested the Terminator exonuclease, but RNA concentration was so low that an RQI could not be calculated, so we did not attempt RNA-seq.

In addition to the ten studies of the wolbachial transcriptome, RNA-seq was applied in five other studies of *Wolbachia* of *D. melanogaster*, *Drosophila ananassae*, *B. malayi*, and *A. albopictus* (Woolfit et al., 2015; Kumar et al., 2016; Luck et al., 2017; Hussain et al., 2023; Cantin et al., 2024).

Woolfit et al. (2015) observed strong differences in wolbachial reads depending on sample type, with cell culture samples yielding the highest coverage of the *Wolbachia* genome. While RNA isolated from fly heads only led to 3% (*w*MelPop) and 1% (*w*MelCS) wolbachial reads, RNA from cell culture samples (*w*MelPop-CLA) led to 8% wolbachial reads when sequenced on GAII and to 18% when sequenced on HiSeq. Among these wolbachial reads, 94–96% corresponded to rRNA genes, except for the sample with 8% wolbachial reads, with a comparably low share of 63% rRNA reads. RNA-seq of RNA from purified *Wolbachia* (*w*MelPop-CLA) led to 83%, the highest percentage of wolbachial reads, with 92% of these reads mapping to *Wolbachia* rRNAs. However, it is unclear whether such a purification procedure affects the transcriptome.

Depending on study design and objectives, purification of *Wolbachia* might be a cost-efficient solution for RNA-seq. Where appropriate, our extracellular culture system of *Wolbachia* could be applied for this purpose (Behrmann et al., 2024), as purification is

performed in the beginning, minimizing the risk of transcriptome alteration before harvest. However, this system has only been developed for the *w*AlbB strain.

Alternative methods for enriching *Wolbachia* mRNA are Cappable-seq and the Agilent SureSelect Kit (Luck et al., 2017; Chung et al., 2019). Cappable-seq, which captures 5'-triphosphorylated RNA, increased wolbachial reads by 4.2-fold compared to untreated total RNA but still yielded only 6.5% wolbachial reads (Luck et al., 2017). The Agilent SureSelect Kit, which uses sequence-specific hybridization probes to capture mRNA, achieved a relatively high proportion of wolbachial reads in gerbil samples (Chung et al., 2019). However, this approach introduces hybridization bias, meaning only pre-selected, well-annotated transcripts are efficiently captured, potentially missing novel or unannotated *Wolbachia* transcripts.

A recent study by Cantin et al. (2024) achieved 1% wolbachial reads for *w*Bm while employing dual RNA-seq. They investigated various rRNA depletion methods and found that DNA probes were most effective, consistent with our findings. Their depletion, however, was RNase H-based, whereas we used a bead-based depletion strategy (riboPOOLs). Importantly, their focus was on enabling dual RNA-seq of *B. malayi* female worms and their *Wolbachia*, while our study aimed to solely increase wolbachial read counts including through the depletion of eukaryotic mRNA. A future comparison of their RNase H-based approach – using DNA probes designed to target *A. albopictus* and wolbachial rRNA, including the *A. albopictus* rRNA pseudogenes described in this study – with our riboPOOL-based strategy would help identify whether enzymatic and bead-based depletion are equally suitable.

Using our custom-designed riboPOOLs in combination with eukaryotic mRNA depletion, we achieved a high level of wolbachial reads (30.2%) together with a reduced amount of rRNA reads (32.0%), offering a powerful approach for RNA-seq studies of *Wolbachia*. We hope that our method for RNA preparation will enable transcriptomic analyses under diverse conditions to further investigate gene regulation in this important endosymbiont and may be adapted for other intracellular bacteria.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

LVB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing
TAH: Data curation, Formal analysis, Writing – original draft, Writing – review & editing
AH: Funding acquisition, Supervision
KN: Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing
KMP: Conceptualization, Funding acquisition, Supervision, Writing – review & editing

Funding

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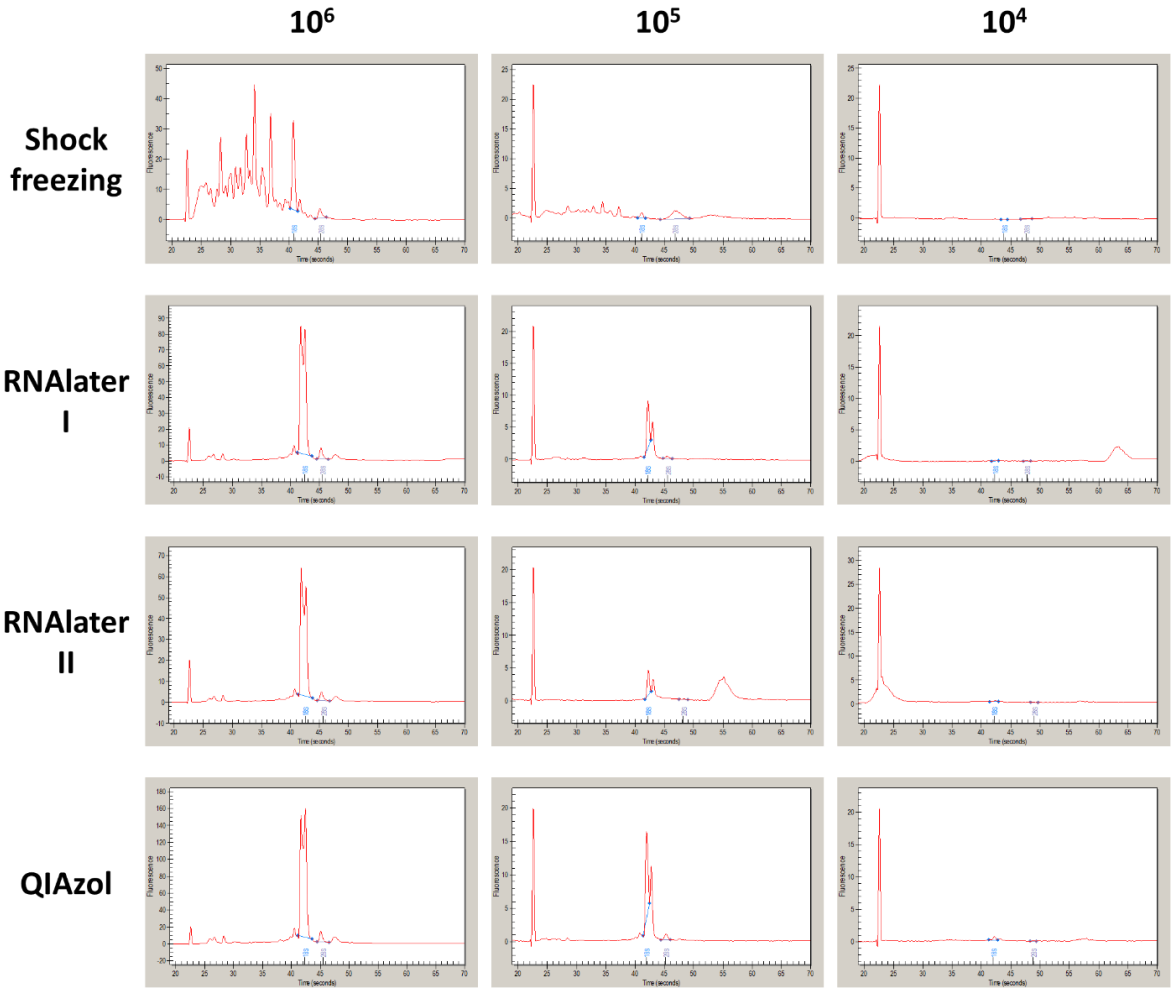
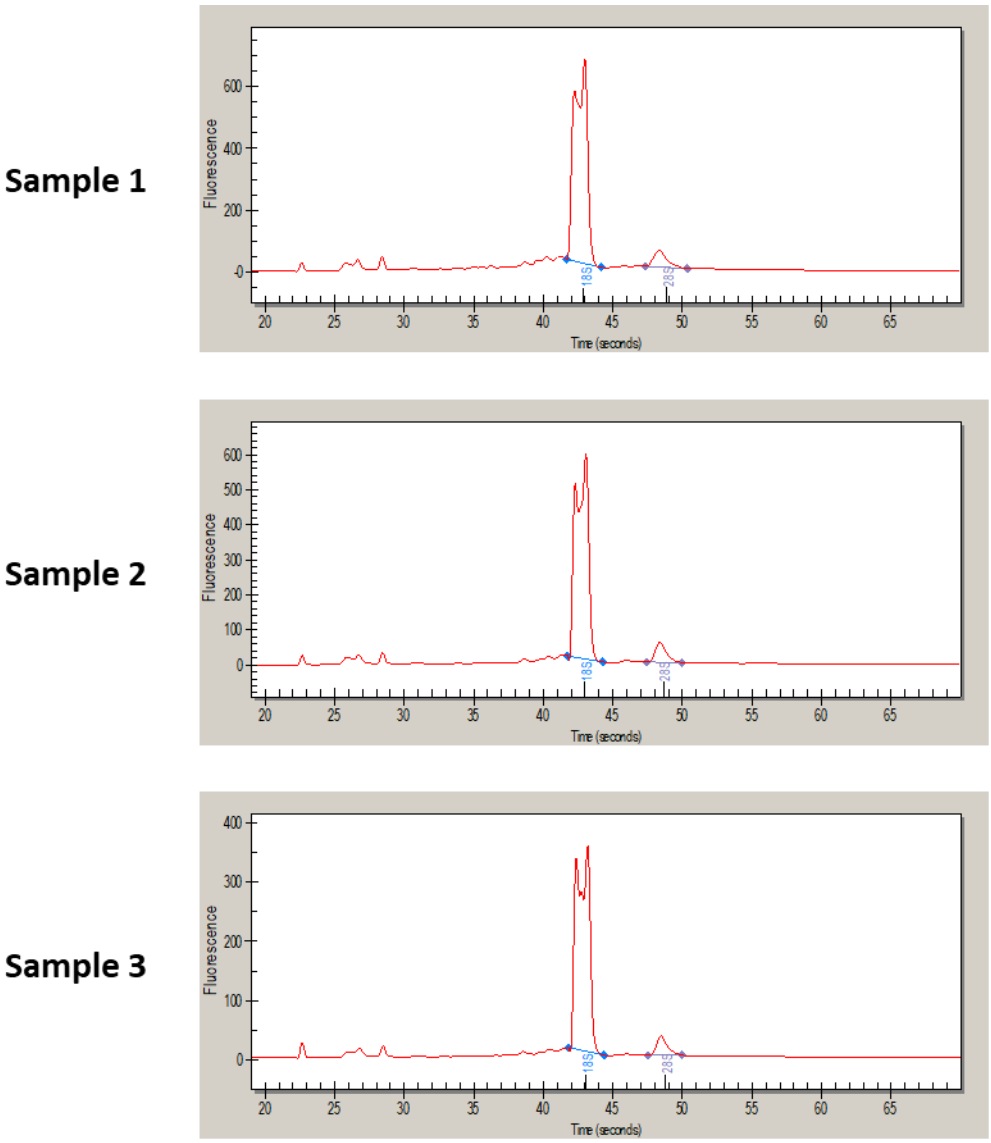
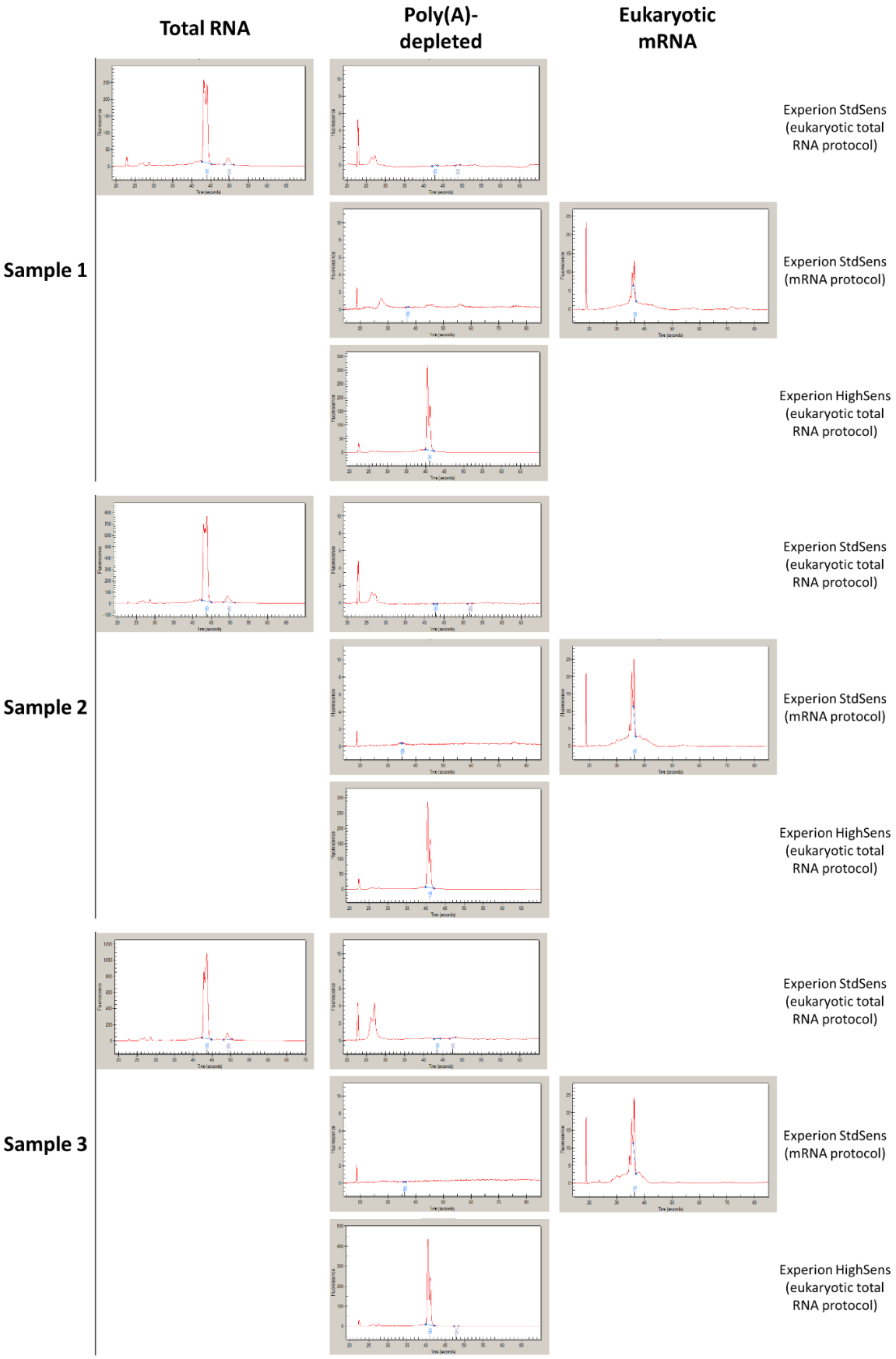
Supplementary Material



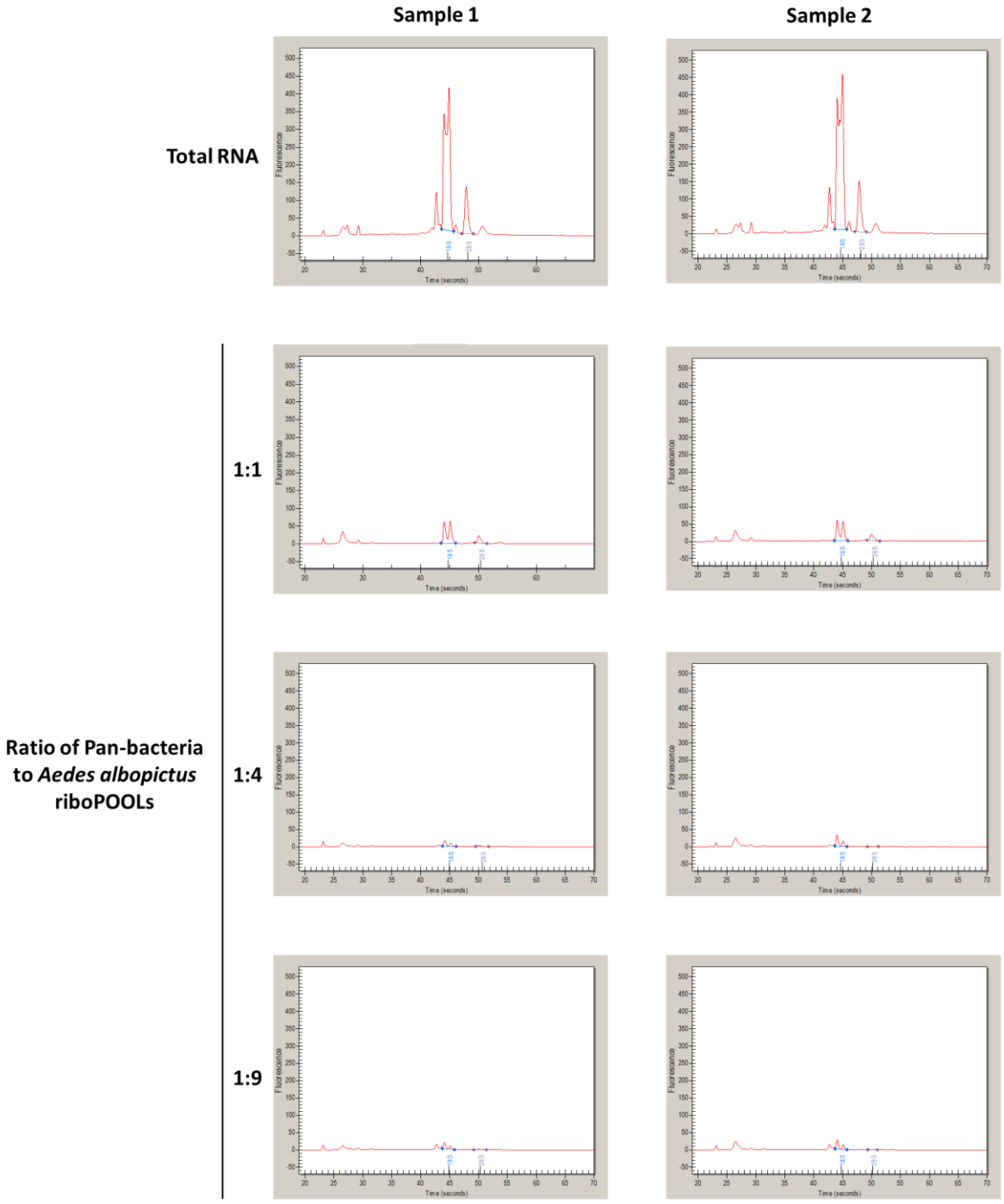
Supplementary Figure 1. Electropherograms of RNA from different preservation methods at varying insect cell concentrations. Insect cells, at concentrations of 6.75 × 10^6, 10^5, and 10^4 cells/ml, were preserved using different methods: shock freezing, RNAlater (I: incubation overnight at 4 °C with an additional washing step before freezing, II: direct freezing), or QIAzol. Total RNA was extracted with the miRNeasy Mini Kit and subsequently analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol on the Experion Automated Electrophoresis Station.



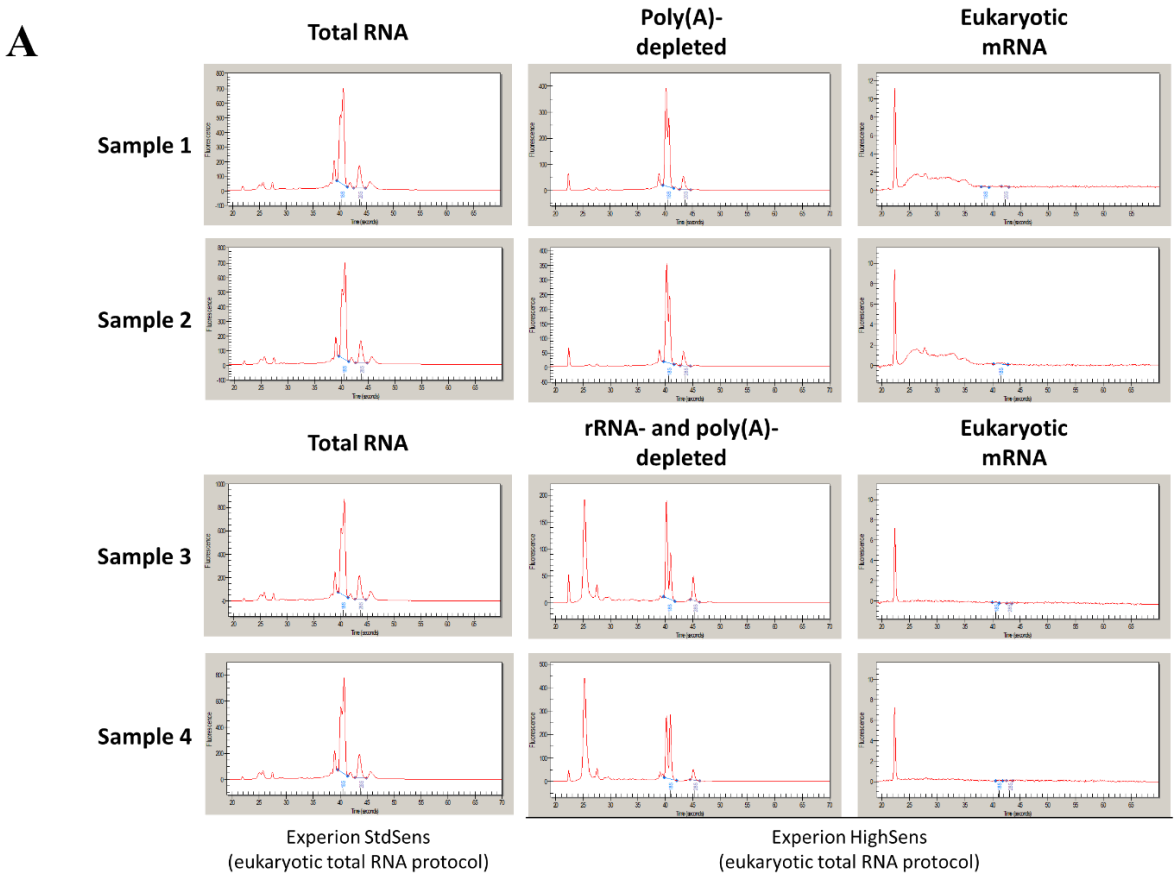
Supplementary Figure 2. Electropherograms of total RNA for RNA-seq. Insect cells were seeded at a density of 0.5×10^7 cells/ml (samples 1, 2) or 0.5×10^5 cells/ml (sample 3) and cultured for 6 days (samples 1, 2) or 9 days (sample 3) in 12-well plates. Total RNA was extracted with the miRNeasy Mini Kit. Analysis was performed with the Experion StdSens Kit using the eukaryotic total RNA protocol on the Experion Automated Electrophoresis Station. The samples were sent for RNA-seq.

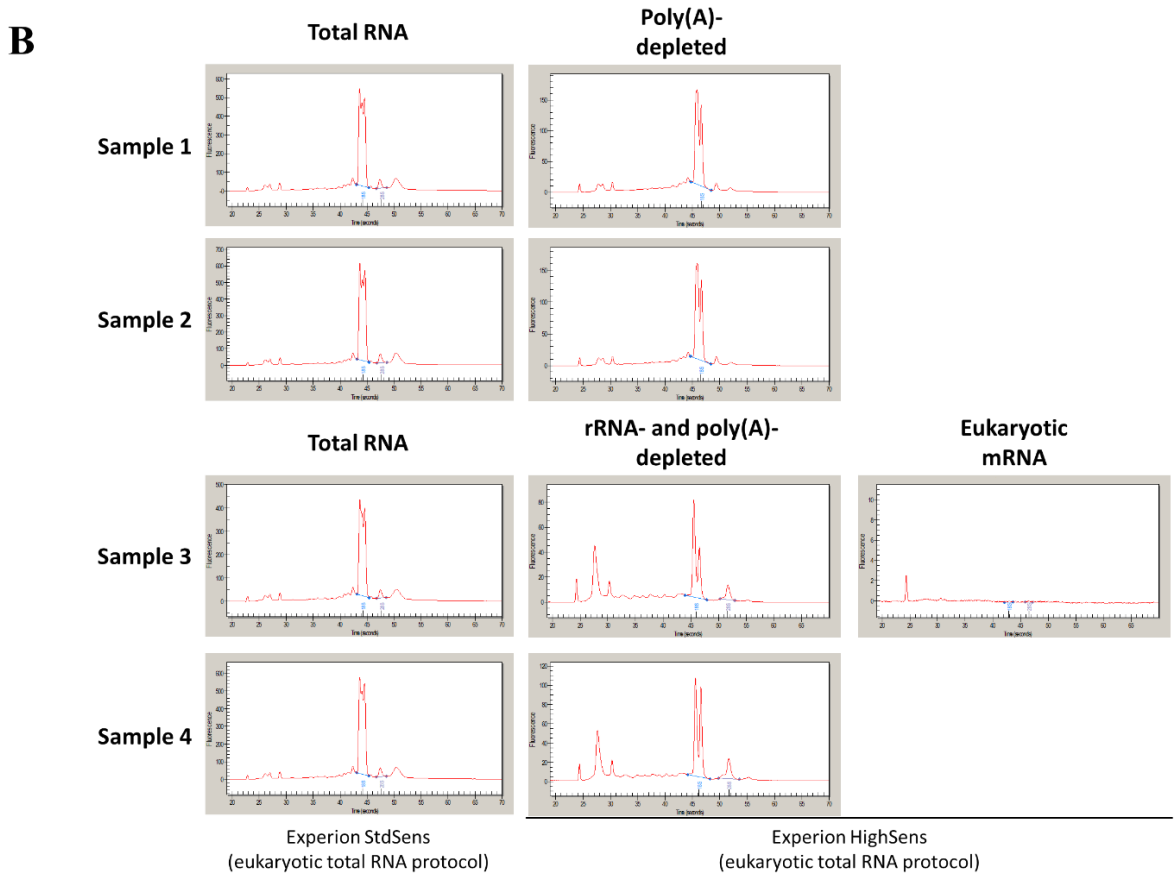

Total RNA
Poly(A)-depleted
Eukaryotic mRNA
Sample 1
Sample 2
Sample 3
Experion StdSens (eukaryotic total RNA protocol)
Experion StdSens (mRNA protocol)
Experion HighSens (eukaryotic total RNA protocol)
Experion StdSens (eukaryotic total RNA protocol)
Experion StdSens (mRNA protocol)
Experion HighSens (eukaryotic total RNA protocol)
Experion StdSens (eukaryotic total RNA protocol)
Experion StdSens (mRNA protocol)
Experion HighSens (eukaryotic total RNA protocol)

Supplementary Figure 3. Electropherograms of poly(A) depletion. Insect cells were seeded at a density of 0.5×10^7 cells/ml and cultured for 6 days (samples 1, 2) or 9 days (sample 3) in 12-well plates. Total RNA was extracted with the miRNeasy Mini Kit. Poly(A) depletion was performed and the resulting poly(A)-depleted RNA and eukaryotic mRNA rescued. The different types of RNA were analyzed on the Experion Automated Electrophoresis Station. First, total RNA and poly(A)-depleted RNA were analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol. The total RNA of samples 1 and 3 was diluted 1:1 before measuring. Second, poly(A)-depleted RNA and eukaryotic mRNA were analyzed with the Experion StdSens Kit using the mRNA protocol. Third, poly(A)-depleted RNA diluted 1:100 was analyzed with the Experion HighSens Kit using the eukaryotic total RNA protocol.

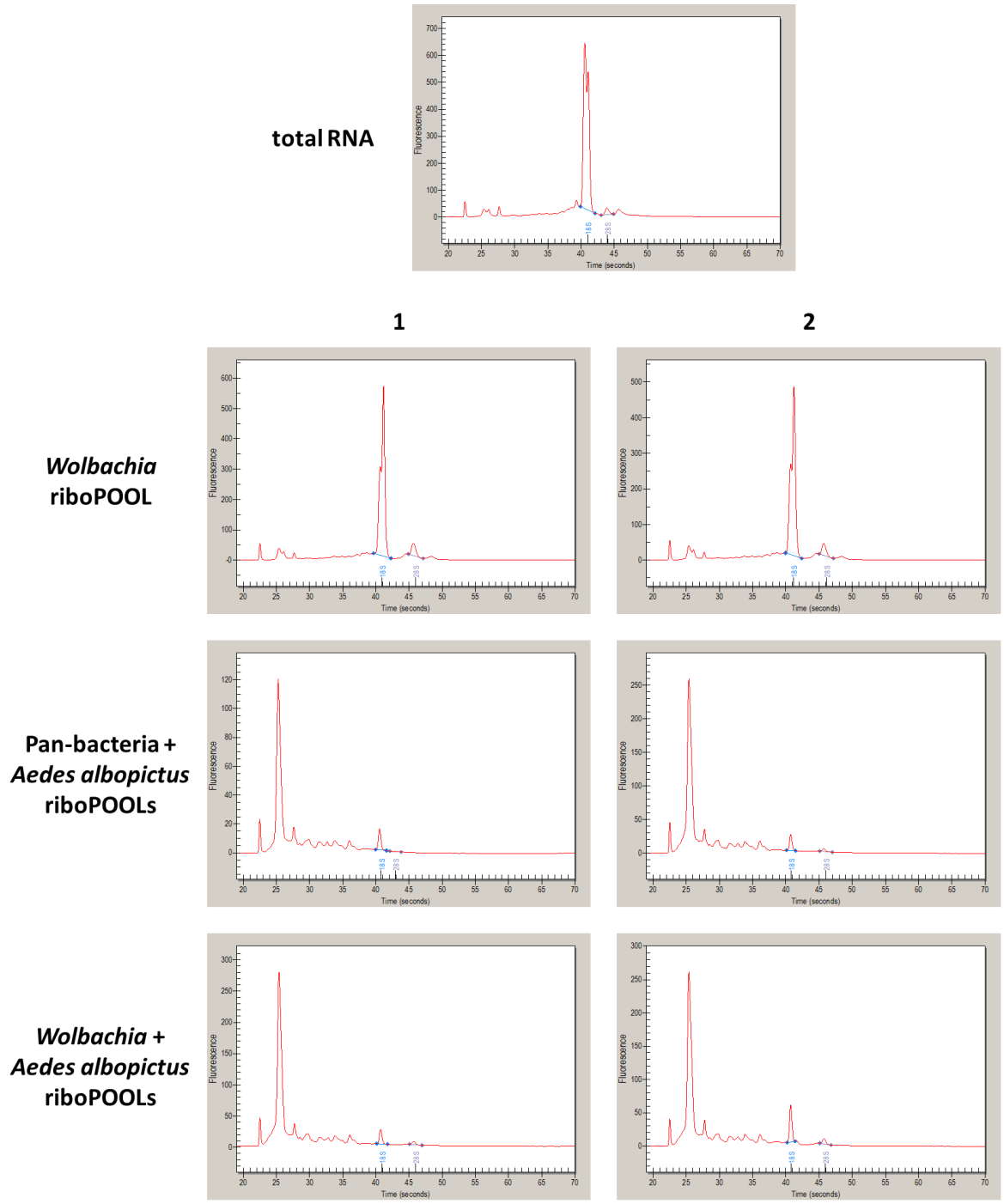


Supplementary Figure 4. Electropherograms showing ratio-dependent depletion of wolbachial and insect cell rRNA by riboPOOLs. Total RNA was extracted with the miRNeasy Mini Kit in technical duplicates and subsequently treated with different ratios of Pan-Bacteria to *Aedes albopictus* riboPOOLs (1:1, 1:4, 1:9) for rRNA depletion. Total RNA and rRNA-depleted RNA were analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol on the Experion Automated Electrophoresis Station.


A
Total RNA
Poly(A)-depleted
Eukaryotic mRNA
Sample 1
Sample 2
Total RNA
rRNA- and poly(A)-depleted
Eukaryotic mRNA
Sample 3
Sample 4
Experion StdSens (eukaryotic total RNA protocol)
Experion HighSens (eukaryotic total RNA protocol)


B
Total RNA
Poly(A)-depleted
Sample 1
Sample 2
Total RNA
rRNA- and poly(A)-depleted
Eukaryotic mRNA
Sample 3
Sample 4
Experion StdSens (eukaryotic total RNA protocol)
Experion HighSens (eukaryotic total RNA protocol)

Supplementary Figure 5. Electropherograms of poly(A)-depleted and rRNA- and poly(A)-depleted RNA. Total RNA was extracted with the miRNeasy Mini Kit from two cell culture flasks of different passages (A, B) in 4 technical replicates each. Poly(A) depletion was performed with Dynabeads. rRNA depletion was performed using a 1:4 ratio of Pan-Bacteria to Aedes albopictus riboPOOLs. Afterwards, poly(A) depletion was performed with Dynabeads. mRNA was rescued as a control and analyzed for the shown samples. All RNA types were subsequently analyzed on the Experion Automated Electrophoresis Station. Total RNA was analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol while the poly(A)-depleted RNA, rRNA- and poly(A)-depleted RNA, and eukaryotic mRNA were analyzed with the Experion HighSens Kit using the eukaryotic total RNA protocol. The poly(A)-depleted RNA was diluted 1:200 (A) or 1:500 (B) and the rRNA- and poly(A)-depleted RNA was diluted 1:2 (A) or 1:10 (B). The depleted samples of B were sent for RNA-seq.



Supplementary Figure 6. Electropherograms showing depletion of wolbachial and insect cell rRNA by different riboPOOLs. Total RNA was extracted with the miRNeasy Mini Kit and subsequently treated with different riboPOOLs (1 µl *Wolbachia* riboPOOL, 2 µl of Pan-Bacteria or *Wolbachia* to *Aedes albopictus* riboPOOLs in a 1:4 ratio) in technical duplicates for rRNA depletion. Total RNA and rRNA-depleted RNA were analyzed with the Experion StdSens Kit using the eukaryotic total RNA protocol on the Experion Automated Electrophoresis Station. There was a slightly insufficient amount of the *Aedes albopictus* riboPOOL available for preparing the second replicate in combination with the *Wolbachia* riboPOOL, though the exact deficit was not quantified.

3 Discussion

Anti-*Wolbachia* therapy is a successful strategy to treat LF and onchocerciasis – both caused by filarial worms that rely on *Wolbachia* for development and reproduction. Despite their importance, *Wolbachia* remain poorly understood, in part, because their obligate intracellular lifestyle has precluded cell-free culture and hindered functional studies. This thesis explored how different antibiotic classes affect *Wolbachia*, with a focus on cellular responses and the potential for resistance development.

To overcome a major experimental limitation, a culture system was established that enabled the replication of *Wolbachia* outside of host cells for the first time (see 2.1). *Wolbachia w*AlbB required a cell lysate fraction from C6/36 insect cells as well as medium with FBS for extracellular replication. Maintenance of *Wolbachia* in the C6/36 cells is dependent on FBS, with some FBS charges leading to a loss of *Wolbachia* over time. This is a disadvantage since the exact composition of FBS is still not known, leading to laborious testing of the suitability of different FBS charges (Baker, 2016; Subbiahanadar Chelladurai et al., 2021). Consequently, it is not surprising that extracellular *Wolbachia* also depend on the presence of FBS. We observed a high variation in replication rates (expressed as fold change from day 0 to day 9) of the cell-free culture, ranging from 2.0 to 14.8 (median: 4.2; mean: 6.4), despite our efforts to maintain consistent conditions. Between the cell-free experiments, we had to switch from one FBS charge to another. Although both were from the same manufacturer, variations between charges could be one reason for the different replication rates observed, since we observed higher rates with the earlier charge. Liu et al. (2023) reported that unidentified small molecules in FBS affect the secretion of the inflammatory factor interleukin-8 and emphasized the need for a uniform standard of quality for FBS to improve the repeatability of experimental results. Another solution would be replacing FBS. The extracellular growth of *C. burnetii* was initially found to be FBS-dependent as well (Omsland et al., 2009), but a defined, FBS-free medium was developed later (Sandoz et al., 2016). This would be a major step for the *Wolbachia* cell-free culture, ensuring greater reproducibility and comparability of experiments. However, since the replication rate of extracellular *Wolbachia* was around 3.5-fold lower than that of intracellular *Wolbachia*, other factors are probably still missing from the cell-free culture.

Their identification would not only lead to greater and sustained extracellular growth, but would also help to further elucidate the *Wolbachia*-host symbiosis.

One idea for such a factor was cholesterol, since related endobacteria, i.e., *Ehrlichia chaffeensis* and *A. phagocytophilum*, were found to depend on cholesterol (Lin and Rikihisa, 2003). All three do not synthesize LPS and might need cholesterol for membrane stability (Lin and Rikihisa, 2003; Wu et al., 2004; Foster et al., 2005). In *w*Mel, a predicted lipase with similarity to a known one with phospholipid-cholesterol acyltransferase activity was conserved (Foster et al., 2005). *A. albopictus* cells can grow without sterols (Mitsuhashi, 1982) but incorporate sterol when grown in FBS (Mitsuhashi et al., 1983). Delipidation of *Wolbachia*-infected C6/36 cells while monitoring *Wolbachia* numbers would allow for testing a putative dependence of *Wolbachia* on cholesterol as was done for viral replication in C6/36 cells by Hafer et al. (2009). There are indications that *Wolbachia* and viruses compete for cholesterol in host cells (Caragata et al., 2013; Edwards et al., 2023). Moreover, *Wolbachia* were found to interfere with cholesterol trafficking in *A. aegypti* and lead to an accumulation of intracellular cholesterol stores (Geoghegan et al., 2017).

While no extension of cell-free *Wolbachia* replication was found upon co-cultivation with insect cell lysate and cholesterol, it should be tested whether cholesterol alone or with reduced levels of insect cell lysate allows for replication of cell-free *Wolbachia*. Like this, it could be tested whether *Wolbachia* have a certain threshold requirement for cholesterol beyond which increases do not lead to greater replication, as was suggested before (Caragata et al., 2013).

So far, it has not been possible to establish a filarial nematode cell line. Other attempts to culture *Wolbachia* of filarial nematodes in insect cells were unsuccessful (Slatko et al., 2014). Therefore, the cultivation of isolated *Wolbachia* of filarial nematodes together with insect cell lysate is unlikely to enable cell-free replication. Even if this approach worked, it would only be a short-term solution and a cell culture would be far more feasible, removing the need to extract filarial nematodes from infected animals for each experiment. Using lysates from the same filarial nematodes for supplementation would also not be suitable, since the lysate would contain *Wolbachia*. As an alternative, lysate from *Wolbachia*-free filarial nematodes such as *A. viteae*, which can be maintained in Mongolian jirds (Johnson et al., 1974), could be explored.

Genetic manipulation of *Wolbachia* as obligate intracellular bacteria has not been possible, hampering the analysis of their biology and host symbiosis. Standard methods such as cloning and antibiotic selection are often not possible for obligate intracellular bacteria (Beare et al., 2011; Beare, 2012). However, the cell-free system might pave the way for genetic manipulation of *Wolbachia*. In *C. burnetii*, cell-free culturing reduced the time needed for genetic transformation from 8–12 weeks to 16 days (Omsland et al., 2011). Recently, CRISPR interference (CRISPRi) was successfully established for cell-free *C. burnetii* cultures for inducible repression of genes (Steiner and Roy, 2024). CRISPRi is a tool for studying gene function by selectively repressing their expression (Qi et al., 2013). Since many of the genes in the reduced genomes of obligate intracellular bacteria are likely essential, gene silencing is a very important tool for their analyses (Ouellette, 2018; Fisher and Beare, 2023). CRISPRi was also established for *Chlamydia* (Ouellette, 2018; Ouellette et al., 2021). While no cell-free *Chlamydia* culture exists, *Chlamydia* can be isolated, transformed with shuttle vectors that contain sequences from their endogenous plasmids, and added to uninfected cells. Others (Rasgon et al., 2006; Nevalainen et al., 2023) and we (see 2.1) have shown that isolated *Wolbachia* are able to infect cells, however, *Wolbachia* were long considered to have no plasmids (Reveillaud et al., 2019). In 2019, a putative plasmid was identified in *Wolbachia* of *Culex pipiens* (Reveillaud et al., 2019) and confirmed to be a widely distributed *Wolbachia* plasmid in *Culex* mosquitoes (Ghousein et al., 2023) that is found in all developmental stages of the investigated mosquitoes (Brunner et al., 2025).

This discovery of a *Wolbachia* plasmid, and being able to culture *Wolbachia* extracellularly – even if currently for only up to 12 days – will help to develop a genetic toolkit for *Wolbachia*. This could be used not only to study the essentiality of conserved PGN genes but also to expand vector control strategies. In the latter, genetic manipulation could be used to insert genes for pathogen-blocking or increased penetrance of cytoplasmic incompatibility (Kaur et al., 2021).

While extracellular growth of *Wolbachia* was only possible for up to 12 days, this system still allows for the testing of antibiotics. We tested the cell wall biosynthesis inhibitors FOS, bacitracin, vancomycin, and ampicillin. FOS-treated extracellular *Wolbachia* displayed the same enlarged cell morphology as previously found for intracellular *Wolbachia* (Vollmer et

al., 2013). We noticed that a phenotypic effect was only observed when we also saw replication in the cell-free system. This indicates that FOS only acts on dividing *Wolbachia*, which is characteristic of cell wall biosynthesis inhibitors. They typically act on dividing bacteria and are ineffective against dormant ones (Sarkar et al., 2017). The enlarged phenotype shows that cell division is then inhibited by FOS.

In an intracellular setting, while cytotoxicity is controlled for via monitoring of the actin gene, we cannot exclude that FOS has some effect on the insect cells that in turn induces cellular effects in *Wolbachia*. However, with confirming the effect in extracellular *Wolbachia*, we can conclude that it is a direct effect of FOS on *Wolbachia*.

The other cell wall biosynthesis inhibitors were not active against extracellular *Wolbachia*. Since bacitracin and vancomycin are relatively large antibiotics that are usually only active in Gram-positive bacteria (Johnson et al., 1945; Sarkar et al., 2017), it was interesting to see whether they would act on extracellular *Wolbachia*. While *Wolbachia* are Gram-negative, they have an unusual cell wall without LPS (Wu et al., 2004; Foster et al., 2005), and with an unknown PGN state which could have made them sensitive to both antibiotics. For example, Gram-negative *N. gonorrhoeae* are sensitive to both bacitracin and vancomycin (Johnson et al., 1945; Windall et al., 1980). Both antibiotics having no effect on extracellular *Wolbachia* confirms the results from cell culture experiments (H. Neufeld, personal communication). Vancomycin binds to the D-Ala-D-Ala dipeptide (Reynolds, 1989). This is synthesized by the ligase Ddl which is expressed (Vollmer et al., 2013) and was found to be inhibited by D-cycloserine in *Wolbachia* (Atwal et al., 2021), showing that the target structure for vancomycin is built in *Wolbachia*. In addition, D-Ala was labeled via click chemistry by Atwal et al. (2021). Although the labeling was not consistently observed (J. Salje, personal communication), this further indicated the presence of the target structure and of a membrane-bound PGN precursor (Atwal et al., 2021). Bacitracin binds to the pyrophosphate moiety of C_{55}-PP (Siewert and Strominger, 1967). Unless lipid II stays membrane-bound in *Wolbachia*, there will be free C_{55}-PP to which bacitracin would bind. The presence of SEDS proteins and PBPs indicates that transglycosylation is possible in addition to transpeptidation, respectively, supporting the hypothesis of free C_{55}-PP in *Wolbachia*. Therefore, we propose that either vancomycin and bacitracin cannot pass

through the outer membrane or are substrates of wolbachial efflux pumps and thus show no effect although the target structures are there.

No effect of ampicillin was expected since *Wolbachia* are resistant against beta-lactams. In previous *in vitro* experiments, different beta-lactams were not effective against *w*AlbB from our group (unpublished data) and *Wolbachia* strains from other studies, including *w*AlbB (Hermans et al., 2001; Fenollar et al., 2003; Fallon, 2018), *w*Mel (Atwal et al., 2021), and *Wolbachia* of *Laodelphax striatellus* (*w*Str) (Fallon, 2018). Similarly, no effect was observed *in vivo* against *Wolbachia* of *L. sigmodontis* (Hoerauf et al., 1999). This was unexpected since *Wolbachia* have conserved 2–3 PBPs, which are expressed in *w*AlbB (Vollmer et al., 2013). Fallon (2018) even reported an increase in *Wolbachia* density upon ampicillin treatment for *w*Str, but not *w*AlbB. The beta-lactam resistance of *Wolbachia* enables use of penicillin in the cell culture medium (Dobson et al., 2002).

With muraymycins, a third class of cell wall biosynthesis inhibitors was found to be effective against *Wolbachia* (see 2.2). Our joint study also found them effective against *C. trachomatis*. For both intracellular bacteria, enlarged cells were observed upon treatment. Interestingly, the same muraymycin derivatives were not active against *Wolbachia* and *Chlamydia*. While MRH-76 and MRH-92 were active against *Wolbachia* and both induced an enlarged phenotype, MRH-92 was the only derivative that did not induce enlarged chlamydial cells. This could be explained by different uptake efficiencies of the compound in the different host cells: *Wolbachia* were cultured in C6/36 insect cells, *C. trachomatis* were cultured in HEp-2 cells.

Many muraymycin derivatives were cytotoxic for the insect cells. Now, with the establishment of our cell-free culture system, we could test their effect and the effect of other antibiotics with known cytotoxicity, such as the MraY inhibitor tunicamycin (Price et al., 2017), on *Wolbachia*. While testing of antibiotics which are cytotoxic might not be relevant for application in the clinic, elucidation of the cellular effects of antibiotics helps investigate the biology of *Wolbachia*. Furthermore, our study has shown that cytotoxicity differs for insect (C6/36) and human cell lines (HEp-2). Since anti-*Wolbachia* therapy is not being developed for *Wolbachia* in insect cells but for humans with filarial infections, the possibility to test antibiotics on *Wolbachia* outside of their insect host cells is a great addition that could hinder the sorting out of compounds which are only cytotoxic for insect

cells. If replication can be reliably sustained, the cell-free culture system would offer a means for mid-throughput susceptibility testing of antibiotics directly on *Wolbachia*.

While the enlarged cell phenotype of *Wolbachia* upon treatment with the cell wall biosynthesis inhibitors FOS and muraymycins resembles ABs of *Chlamydia* or *W. chondrophila*, it is not known whether all criteria of ABs are fulfilled. Although the cells are enlarged and cell division is inhibited, it is not known whether this phenotype of *Wolbachia* is reversible, which would make them persistent ABs (Wyrick, 2010). Moreover, penicillin-induced ABs in *Chlamydia* are polyploid (Lambden et al., 2006; Skilton et al., 2009), and, likewise, FOS-induced ABs in *W. chondrophila* have multiple genome copies (Scherler et al., 2020). Future research may clarify whether these enlarged *Wolbachia* can revert to normal cell size and whether they are polyploid as well.

As with *Wolbachia*, *Chlamydia* were long thought to be cell wall-less; however, this has changed in the past decade. It was revealed that a transient septal PGN ring is important for their cell division, explaining why cell wall biosynthesis inhibitors affect *Chlamydia* (Liechti et al., 2014; Packiam et al., 2015; Liechti et al., 2016). A similar finding, which would solve the wolbachial anomaly, is still pending for *Wolbachia*. Atwal et al. (2021) did not observe such a ring or any particular structure in their D-Ala staining. This could have several reasons: (i) it might be that there is a septal PGN ring in *Wolbachia* but that it was not detected. In contrast to *Chlamydia*, not much is known about the wolbachial developmental cycle. Since *Wolbachia* replicate slowly – we determined a 14.8-fold increase in cell culture after 9 days (see 2.1), corresponding to 0.43 cell divisions per day assuming exponential growth – the right time point might have been missed. (ii) However, it could also be that there is no septal ring but a rudimentary PGN structure, possibly with only one layer that might even have gaps. This would explain why the staining is only punctuate. As *Wolbachia* kept FtsZ, they might not depend on a septal PGN ring like *Chlamydia* do that have lost FtsZ. (iii) Another possibility would be that the integration of the modified D-Ala was not very efficient, so that a structure could not be detected. Atwal et al. (2021) used a modified single D-Ala, ethynyl-D-alanine (EDA). Incorporation of EDA was also not possible for *C. trachomatis* (Liechti et al., 2014), therefore using the modified dipeptide EDA-DA could be feasible. As *Wolbachia* are sensitive to D-cycloserine, this could be added to the culture, which – through inhibiting the synthesis of D-Ala-D-Ala dipeptide –

could prompt them to integrate the modified dipeptide EDA-DA used for click chemistry (Liechti et al., 2014). To verify that EDA or EDA-DA is incorporated into *Wolbachia*, two negative controls, which were missing in the study by Atwal et al. (2021), need to be included: first, a control with uninfected host cells to account for any non-specific incorporation of the respective probe; and second, a control with the modified L-enantiomer, ELA or ELA-LA, respectively, to confirm the specificity for the D-enantiomer usually found in PGN (Liechti et al., 2014). Future research will aim to optimize the incorporation of EDA or EDA-DA into *Wolbachia*, which may allow for the detection of a PGN ring and advance our understanding of the cell wall composition of *Wolbachia*.

The standard method to determine the presence and structure of PGN is UPLC-MS (Kühner et al., 2014; Alvarez et al., 2020). A first attempt for a member of Chlamydiae, *W. chondrophila*, was unsuccessful (Jacquier et al., 2015a), but a second attempt with more material led to the confirmation of PGN in *W. chondrophila* (Jacquier et al., 2019). They were able to synchronize the cell cycle of *W. chondrophila*, ensuring that the bacteria are at the same metabolic and replicative stage. Synchronization of *Wolbachia* could facilitate the successful extraction of sufficient quantities of their putative PGN. The characteristics of the Alphaproteobacterium *O. tsutsugamushi* are similar to *Wolbachia* with regard to PGN, i.e., the conserved genes and effects of FOS and D-cycloserine but not beta-lactams. Here, the isolation of PGN for MS analysis has not been possible (Atwal et al., 2017). As for *W. chondrophila*, the amount of cell culture of *O. tsutsugamushi* probably needs to be scaled up to enable PGN detection. Similarly, large amounts are likely required to detect the putative PGN of *Wolbachia*.

Although further research is needed to investigate the putative PGN of *Wolbachia*, our findings that FOS and muraymycins, which target the first dedicated cytoplasmic and the first membrane-bound step of PGN biosynthesis, respectively, are active against *Wolbachia* and induce an enlarged phenotype confirm the important function of lipid II in *Wolbachia*.

As PGN and its precursor lipid II are often referred to as the Achilles' heel of bacteria (Breukink and de Kruijff, 2006; Schneider and Sahl, 2010; Sibinelli-Sousa et al., 2021) and the obligate symbiotic relationship with *Wolbachia* was referred to as the Achilles' heel of filarial nematodes (Hoerauf and Pfarr, 2007), this prompts the question whether the wolbachial PGN or lipid II are then the Achilles' heel of *Wolbachia* and thus filarial

nematodes. While lipid II is definitively important for *Wolbachia*, the concentrations of antibiotics needed for depletion of *Wolbachia* are high and not feasible for application in the clinic. For FOS, 512 µg/ml are required to induce the enlarged cells (Vollmer et al., 2013). A concentration of 32 µg/ml only slightly inhibited growth of *w*AlbB and, according to our definition (*16S rRNA*/*act* ratio <0.5, see 2.3), the MIC is >128 µg/ml, with 128 µg/ml only leading to a 5-fold reduction (Henrichfreise et al., 2009). Further, we do not know if the enlarged cells represent a persistence phenotype as previously discussed. In contrast to targeting PGN biosynthesis, inhibiting transcription via CorA or rifampicin or inhibiting translation via DOX have been shown to be effective strategies for the treatment of filarial diseases. Here, low concentrations of 1 µg/ml (CorA, rifampicin) and 4 µg/ml (DOX) depleted *w*AlbB >15-fold *in vitro*. Still, studying the wolbachial cell wall provides insights into bacterial evolution, the minimal requirements for building a putative PGN, and has implications for antibiotic targeting as well as immune system interactions.

New antifilarial drugs with macrofilaricidal activity are needed. The antibiotic CorA is a promising candidate, and we therefore investigated the resistance development against CorA in *Wolbachia* in a long-term cell culture assay (see 2.3). The frequency of mutation to resistance refers to how often mutations that lead to resistance occur in a population and is usually determined by exposing the population to 4 × MIC of a drug. In our study, we did not observe any resistance development at the selected concentrations, including 4 × MIC of CorA, so we were unable to calculate an exact frequency. However, since 2.75×10^9 *Wolbachia* per group were treated and no loss of sensitivity was observed, we estimated that the frequency of mutation to resistance must be lower than the inverse of the number of bacteria, which is $<2.75 \times 10^{-9}$. Typically, a frequency $<10^{-9}$ is considered acceptable for clinical use of antibiotics. For future experiments, including sequencing of the genes of RNA polymerase or whole genome sequencing would provide further information about putative mutations that have developed, as was done for CorA resistance studies in *S. aureus* and *N. gonorrhoeae* (Balansky et al., 2022; Balthazar et al., 2024).

In general, resistance development of *Wolbachia* is not known. Richardson et al. (2012) estimated substitution rates per site for each codon position (1st, 2nd, and 3rd) in *Wolbachia* per *D. melanogaster* generation and assumed 10 generations per year. Based on these values, the mean substitution rate across all codon positions is calculated as

6.57×10^{-9} substitutions/site/year. For different methicillin-resistant *S. aureus* strains, rates have been determined to 1.3×10^{-6} and 2×10^{-6} substitutions/site/year (Nübel et al., 2010; Holden et al., 2013). For *N. gonorrhoeae*, determination of the exact rate is complicated by frequent homologous recombination; however, estimates suggest rates of 4.54×10^{-6} and 4.75×10^{-6} substitutions/site/year (Golparian et al., 2020; Joseph et al., 2022). In comparison, the estimated mean substitution rate for *Wolbachia* is more than 1,000-fold lower. Although this is only an approximation, the low rate supports the idea that resistance against CorA is likely to develop slowly in *Wolbachia*.

RNA-seq can help elucidate the cellular effects of antibiotics (Chernov et al., 2019). We have established an improved workflow for RNA preparation for RNA-seq of *Wolbachia* using custom-designed riboPOOLs for rRNA depletion and Dynabeads for eukaryotic mRNA depletion. This led to 30.2% wolbachial reads, the highest percentage ever achieved for intracellular *Wolbachia* (see 2.4). Only one other study achieved a similar percentage (29.7%) for *Wolbachia*; however, the kit they used was discontinued (Leitner et al., 2021). In one study, *Wolbachia* were isolated from cell culture immediately before RNA extraction (Woolfit et al., 2015). While this efficiently removed host cell rRNA and mRNA, it may have led to changes in the wolbachial transcriptome as the endobacteria responded to the change in their environment. In contrast, with our cell-free method, *Wolbachia* isolation can be separated from RNA extraction by several days. In this case, *Wolbachia* would first be isolated and then grown for up to 12 days before RNA extraction, potentially allowing them to recover from the initial stress of isolation and return to a more natural transcriptional state. Another option would be to add an RNA polymerase inhibitor such as rifampicin before isolation to prevent changes to the transcripts. This might be more error-prone and requires optimization of conditions to inhibit transcription. Since rifampicin could also have cellular effects before transcription is stopped, this could modify the results.

RNA-seq could be applied for FOS-treated *Wolbachia*. FOS is particularly interesting because it is known to synergize with many other antibiotics and affects several pathways in addition to cell wall biosynthesis in free-living bacteria with a classical PGN (Petek et al., 2010; Falagas et al., 2016; Marquès et al., 2020; Turner et al., 2020; Gil-Gil et al., 2021). In *Wolbachia*, where the PGN pathway is highly reduced and not fully characterized, RNA-seq

could provide insight into whether FOS influences additional pathways beyond cell wall biosynthesis as well and help elucidate the transcriptional changes associated with the inhibition of lipid II synthesis.

Regarding onchocerciasis, ophthalmologist Sir Harold Ridley called it “inconceivable that in the end science will be defeated by a filaria” (Ridley, 1945). 80 years of scientific efforts have passed and, although we still have challenges to meet, Ridley’s optimism can be shared. This thesis contributes to the understanding of antibiotic-induced cellular effects in *Wolbachia*, with implications for their PGN status and development of a new antibiotic. The methods developed, cell-free cultivation and improved preparation of RNA for RNA-seq, will help in the ongoing investigation of this important endosymbiont. Further research is needed to understand *Wolbachia*’s cellular response to cell wall biosynthesis inhibitors and whether the enlarged phenotype represents a persistence-like state. This knowledge will enhance our grasp of cell morphogenesis and integrity in these endobacteria and open avenues for the discovery of novel anti-wolbachial targets that, like CorA, could be developed into new treatment options for filarial infections in humans.

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Scientific Contributions

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Poster presentation at the 2nd International ANTIBIOTIC CellMAP Symposium, Bad Boll, Germany, 2023 (“Cellular effects of fosfomycin on *Wolbachia*”)

Oral presentation at the TRR261 Meeting, Bonn, Germany, 2022 (“Mode of action of cell wall biosynthesis inhibitors in *Wolbachia* spp.”)

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Poster presentation at the 5th Interdisciplinary Course on Antibiotics and Resistance, Veyrier-du-Lac, France, 2021 (“Mode of action of cell wall biosynthesis inhibitors in *Wolbachia* spp.”)

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Appendix

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In vitro extracellular replication of *Wolbachia* endobacteria

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Obligate intracellular endobacteria of the genus *Wolbachia* are widespread in arthropods and several filarial nematodes. Control programs for vector-borne diseases (dengue, Zika, malaria) and anti-filarial therapy with antibiotics are based on this important endosymbiont. Investigating *Wolbachia*, however, is impeded by the need for host cells. In this study, the requirements for *Wolbachia w*AlbB growth in a host cell-free *in vitro* culture system were characterized via qPCRs. A cell lysate fraction from *Aedes albopictus* C6/36 insect cells containing cell membranes and medium with fetal bovine serum were identified as requisite for cell-free replication of *Wolbachia*. Supplementation with the membrane fraction of insect cell lysate increased extracellular *Wolbachia* replication by 4.2-fold. Replication rates in the insect cell-free culture were lower compared to *Wolbachia* grown inside insect cells. However, the endobacteria were able to replicate for up to 12 days and to infect uninfected C6/36 cells. Cell-free *Wolbachia* treated with the lipid II biosynthesis inhibitor fosfomycin had an enlarged phenotype, seen previously for intracellular *Wolbachia* in C6/36 cells, indicating that the bacteria were unable to divide. In conclusion, we have developed a cell-free culture system in which *Wolbachia* replicate for up to 12 days, providing an *in vitro* tool to elucidate the biology of these endobacteria, e.g., cell division by using compounds that may not enter the C6/36 cells. A better understanding of *Wolbachia* biology, and in particular host-symbiont interactions, is key to the use of *Wolbachia* in vector control programs and to future drug development against filarial diseases.

KEYWORDS

***Wolbachia*, cell-free, endosymbionts, intracellular bacteria, *in vitro* culture, filariasis, vector control**

1 Introduction

Wolbachia are intracellular Gram-negative alpha-proteobacteria found in arthropods and in some nematodes, including filarial nematode species pathogenic to humans (Taylor and Hoerauf, 2001; Fenn et al., 2006; Zug and Hammerstein, 2012). They reside in host-derived vesicles within cells of somatic tissues as well as the host germline through which they are transmitted vertically from the mother to the offspring (Casiraghi et al., 2007; Serbus and Sullivan, 2007). A common feature of endosymbiotic bacteria is the reduction of genome size due to the evolutionary adaptation to their host (Stepkowski and Legocki, 2001). This is also the case for *Wolbachia*, which possess a limited metabolic capacity. They lack almost all biosynthetic pathways to produce amino acids *de novo* and have retained almost only incomplete pathways for the synthesis of vitamins and cofactors, all of which are most probably

provided by their host (Wu et al., 2004; Foster et al., 2005; Slatko et al., 2010).

Wolbachia endosymbionts of arthropods are largely facultative and often exhibit a parasitic association with their hosts (Werren et al., 2008). The stability of *Wolbachia* transmission is ensured by reproductive manipulations such as male-killing, feminization, parthenogenesis, and cytoplasmic incompatibility between infected and uninfected organisms (Fenn and Blaxter, 2006). Of note, benefits of an infection with *Wolbachia*, e.g., in terms of protection against different pathogens, have been reported (Hedges et al., 2008; Teixeira et al., 2008; Kambris et al., 2009; Moreira et al., 2009). Here, especially anti-viral effects have gained great interest as *Wolbachia* could be used to control vector-borne human diseases such as dengue fever (Blagrove et al., 2012; Velez et al., 2023).

Wolbachia of filarial nematodes are, in contrast to *Wolbachia* of arthropods, intrinsically tied to their host. Here, they are mutualistic endosymbionts that depend on compounds produced by the host, but in turn are believed to provide metabolites that cannot be synthesized by the nematodes *de novo*, e.g., heme, purines, pyrimidines, FAD, and riboflavin, essential for worm survival (Wu et al., 2004; Foster et al., 2005; Slatko et al., 2010). It was demonstrated that *Wolbachia* depletion by the antibiotic doxycycline leads to block in development, sterility, and death of adult filarial worms (Hoerauf et al., 2001; Taylor et al., 2012). Thus, filarial *Wolbachia* are an effective target for anti-filarial therapy.

The cultivation of *Wolbachia* as obligate intracellular bacteria is challenging. To date, filarial *Wolbachia* cannot be cultured *in vitro* (Slatko et al., 2014) and only a few culture systems exist, in which insect cell lines are stably infected with *Wolbachia* strains from arthropods (Fenollar et al., 2003a; McMeniman et al., 2008; Conceição et al., 2021). In these culture systems, *Wolbachia* are protected from the environment by at least three lipid membrane barriers: the insect cell membrane, vesicle membrane, and the *Wolbachia* cell membranes. Therefore, molecular biology techniques, e.g., genetic transformation, cannot be applied. Additionally, many molecules cannot pass the insect cell membrane, which hampers the elucidation of *Wolbachia* biology and its symbiosis with the host cell.

However, since *Wolbachia* are transmitted from somatic tissue to the germline (Frydman et al., 2006; Landmann et al., 2012), and also horizontally between host species (Dyson et al., 2002; White et al., 2017), even with plants as temporary hosts (Li et al., 2017), they require an extracellular stage (Nevalainen et al., 2023). This stage has been observed in the hemolymph of insects, foregut of ants, and pseudocoelomic cavity of filarial nematodes (Fischer et al., 2011; Andersen et al., 2012; Frost et al., 2014). Rasgon et al. (2006) showed that *Wolbachia* purified from insect cells could be maintained in cell-free culture medium for at least 1 week without loss of viability or infectivity. More recently, the metabolic activity of extracellular *Wolbachia* was measured via phenotypic microarrays over 4 days (Krafsur et al., 2020). However, *Wolbachia* in these cultures did not replicate outside the insect cell (Rasgon et al., 2006; Krafsur et al., 2020).

For a few intracellular bacteria, e.g., *Coxiella burnetii*, *Chlamydia trachomatis*, *Ehrlichia chaffeensis,* and *Anaplasma phagocytophilum*, cell-free culture systems were developed that support metabolic activity (Omsland et al., 2008, 2012; Eedunuri et al., 2018; Zhang et al., 2021). After further modifications, cell-free growth of *Coxiella burnetii* was made possible, accelerating genetic transformation (Omsland et al., 2009, 2011). An adapted medium allows for the non-antibiotic-based selection of genetic transformants (Sandoz et al., 2016).

In this study, we provide first evidence of *Wolbachia* replication in a host cell-free *in vitro* culture. Growth of *Wolbachia w*AlbB was observed when the medium was supplemented with total lysate from *Aedes albopictus* C6/36 insect cells. Furthermore, we could show that the necessary components for the replication of the endobacteria in cell-free medium are contained in the membrane fraction of the insect cell lysate and in fetal bovine serum (FBS).

2 Materials and methods

2.1 C6/36 insect cell culture

The *Aedes albopictus* C6/36 insect cell line, uninfected or infected with the *Wolbachia pipientis* supergroup B strain of *Aedes albopictus* (*w*AlbB), were cultured as previously described (Turner et al., 2006; Henrichfreise et al., 2009). Infected and uninfected C6/36 cells were grown at 26°C in 75 cm^2 culture flasks (Greiner, Frickenhausen, Germany) with 15 mL standard medium consisting of Leibovitz's L15 medium (Thermo Fisher Scientific, Waltham, Massachusetts, United States) supplemented with 5% fetal bovine serum (FBS; PAA Laboratories, Cölbe, Germany or PAN-Biotech, Aidenbach, Germany), 1% MEM non-essential amino acids (PAA Laboratories or Thermo Fisher Scientific), 2% tryptose phosphate broth (Sigma-Aldrich, Steinheim, Germany) and 1% penicillin/streptomycin (PAA Laboratories or Thermo Fisher Scientific). The standard 5% FBS in the culture media was changed to 20% to increase the percentage of infected cells (Clare et al., 2015) for later experiments as indicated.

2.2 Isolation of *Wolbachia* from insect cells

Wolbachia were purified from infected C6/36 cells either as described by Rasgon et al. (2006) or by an abbreviated protocol. The C6/36 cells were grown to ~90% confluence. Cells were harvested with a cell lifter (Corning, New York, United States) in 10 mL standard medium and lysed by vortexing with 100 sterile 3 mm borosilicate glass beads (Sigma-Aldrich) for 5 min. Cell debris was removed by centrifugation at 2,500 g for 10 min at 4°C (Heraeus Multifuge 4 KR, Heraeus, Hanau, Germany) and the supernatant was filtered through a 5 µm syringe filter (Sartorius, Göttingen, Germany). Our abbreviated protocol ended here, so that the insect cell lysate remained in the suspension. For purification following the procedure of Rasgon et al. (2006), *Wolbachia* were pelleted from the filtered supernatant by centrifugation at 18,400 g for 5 min at 4°C (Eppendorf Centrifuge 5,424 R, Eppendorf, Hamburg, Germany) on a 250 mM sucrose cushion (Sigma-Aldrich) and suspended in 10 mL standard medium. In contrast to Rasgon et al. (2006), the subsequent filtration was not performed with a 2.7 µm filter, but with a 1.2 µm syringe filter (Sartorius). The genomic DNA (gDNA) was isolated and the number of *Wolbachia* was determined by quantitative real-time PCR (qPCR) of the single-copy *Wolbachia* 16S rRNA gene as previously described (Makepeace et al., 2006).

2.3 Cell-free *Wolbachia* culture

To investigate the effect of insect cell lysate (see below) on isolated *Wolbachia,* the bacteria were purified from C6/36 either using the

procedure published by Rasgon et al. (2006) or by the abbreviated procedure in which the insect cell lysate was retained. Isolated *Wolbachia* were diluted 1:5 in standard medium and incubated in 25 cm^2 plug-sealed cell culture flasks (Greiner) at 26°C for 15 days. The number of *Wolbachia* was determined by 16S rRNA gene qPCR every one to three days. In the following assays, 200 µL cell-free *Wolbachia* extracted by the abbreviated protocol were incubated in F-bottom 96-well plates (Greiner) at 26°C for 12 days, and *Wolbachia* numbers were quantified by qPCR on day 0 and subsequently every three days.

For insect cell lysate titration assays, isolated *Wolbachia* (0.5–1.5 × 10^3 16S rRNA gene copies/µL) were added to total insect cell lysate equivalent to final concentrations of 0.95 × 10^6 cells/mL, 1.9 × 10^6 cells/mL, or 3.8 × 10^6 cells/mL uninfected C6/36 cells as counted prior to lysis. Dilutions were prepared in standard medium. For *Wolbachia* cell number titration assays, different amounts of *Wolbachia* ranging from 10^2 to 10^5 16S rRNA gene copies/µL were diluted in total cell lysate prepared from 0.95 × 10^6 uninfected C6/36 cells and standard medium.

2.4 Preparation of insect cell lysate

2.4.1 Total insect cell lysate

Insect cell lysate was generated from uninfected C6/36 cells. Briefly, cells were harvested in 10 mL standard medium and the amount of uninfected C6/36 cells was calculated using a Neubauer counting chamber (Laboroptik, Bad Homburg, Germany). Then, C6/36 cells were lysed by vortexing with 100 sterile 3 mm 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.

2.4.2 Fractionation of insect cell lysate

Total insect cell lysate was fractionated by centrifugation at 20,000 g for 30 min at 4°C (Eppendorf Centrifuge 5,424 R) or at 100,000 g for 1 h at 4°C (Sorvall Discovery M120 SE, Sorvall, Waltham, USA), respectively. The supernatants containing microsomes and plasma membranes (Fraction 1) or the soluble cytoplasmic content (Fraction 3), respectively, were retained. Since ultracentrifugation could not be performed under sterile conditions, the supernatant obtained after centrifugation at 100,000 g for 1 h was sterile filtered through a 0.2 µm syringe filter (Sartorius), and the pellet was discarded. The pellet obtained after centrifugation at 20,000 g for 30 min containing nuclear debris and large organelles (Fraction 2) was dissolved in the same volume of standard medium as the starting volume of total lysate. Fractions were used for the preparation of cell-free *Wolbachia* cultures with a concentration of 0.5–1 × 10^3 16S rRNA gene copies/µL. The final concentration of fraction added to the culture was equivalent to 0.95 × 10^6 C6/36 cells/mL as counted prior to lysis. For testing combinations of fractions, the final concentration of each fraction was 0.95 × 10^6 cells/mL, and standard medium with 20% FBS was used. *Wolbachia* cultures with fractions were incubated at 26°C for 12 days. When supplementation with freshly prepared Fraction 1 on day 9 was tested, standard medium with 20% FBS was used, and growth was monitored until day 15.

2.4.3 Insect cell lysate with and without FBS

Wolbachia were purified as described above. Two different insect cell lysates were prepared in cell culture medium with and without FBS. Prior to the preparation of insect cell lysate without FBS, the C6/36 cells were washed once in cell culture medium lacking FBS. Both lysates were centrifuged at 20,000 g for 30 min at 4°C and the supernatants were retained (Fraction 1). *Wolbachia* cultures containing Fraction 1 with and without FBS were incubated at 26°C for 12 days. A control containing *Wolbachia* incubated only in standard medium with FBS was included. The initial *Wolbachia* concentration was 0.1–1 × 10^4 16S rRNA gene copies/µL and the final concentration of Fraction 1 was equivalent to 0.95 × 10^6 cells/mL.

2.5 Supplementation of cell-free culture with cholesterol

Cell-free *Wolbachia* cultures were prepared as described above with 0.5–1 × 10^3 16S rRNA gene copies/µL isolated *Wolbachia* and Fraction 1 from insect cell lysate equivalent to 0.95 × 10^6 cells/mL diluted in standard medium with 20% FBS, cultured in 96-well plates at 26°C and supplemented with 0.1 or 1 mg/mL water-soluble cholesterol (Sigma-Aldrich) for 12 days.

2.6 Infection of C6/36 insect cells with *Wolbachia* from cell-free culture

Cell-free *Wolbachia* cultures were prepared as described above with 0.5 × 10^3 16S rRNA gene copies/µL isolated *Wolbachia* and Fraction 1 from insect cell lysate equivalent to 0.95 × 10^6 cells/mL diluted in standard medium and cultured in 96-well plates at 26°C for 12 days. After 9 days, uninfected C6/36 cells were seeded in an F-bottom 24-well plate (Greiner) with 10^5 cells/well in triplicate. On day 12, the medium was removed from the uninfected C6/36 cells and 750 µL of the cell-free *Wolbachia* culture were added, corresponding to a multiplicity of infection (MOI) of 14. As a negative control, *Wolbachia* were heated at 95°C for 10 min, before adding them to the uninfected C6/36 cells. The 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 overnight. On the next day, cells were transferred into an F-bottom 6-well plate (Greiner) containing 1.5 mL standard medium with 10% FBS and incubated at 26°C. After 6 days, the C6/36 cells were harvested in fresh standard medium and transferred into an 8-well culture slide (BD Falcon, Corning, United States). Additionally, samples were taken for qPCR. C6/36 cells were grown on culture slides for 1 day. *Wolbachia* infection was subsequently examined by immunofluorescence microscopy using rabbit anti-*w*PAL primary antiserum (1:1,000 in PBST; Taylor Laboratory, Liverpool School of Tropical Medicine, Liverpool, UK) and a goat anti-rabbit Alexa 488-conjugated secondary antibody (1:200 in PBST; Thermo Fisher Scientific) and counterstained with 0.25 µg/mL DAPI (Sigma-Aldrich) as described previously (Turner et al., 2009; Vollmer et al., 2013). Cells were then analyzed with a Zeiss Axio Observer.Z1 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) at the respective wavelengths.

2.7 Quantitative real-time PCR

gDNA was extracted from 200 µL using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for DNA purification from blood or body fluids with an adjusted elution volume of 50 µL in a QIAcube robotic workstation (Qiagen). *Wolbachia* cell numbers were calculated by quantification of 16S rRNA gene copies by qPCR as previously described (Makepeace

et al., 2006) using the HotStar Taq Polymerase Kit (Qiagen). A qPCR reaction contained 1x HotStar Taq polymerase buffer, 3 mM $MgCl_2$, 200 µM dNTPs, 0.2 µL SYBR Green (1,000-fold diluted in DMSO; Fermentas, St. Leon-Rot, Germany), 0.5 µM 16S rRNA primers (forward: 5'-TTGCTATTAGATGAGCCTATATTAG-3′, reverse: 5'-GTGTGGCTGATCATC CTCT-3′; Microsynth, Balgach, Switzerland), 0.5 U HotStar Taq polymerase and 2 µL of extracted gDNA (1:20 diluted in AE buffer for cell culture samples, undiluted for cell-free samples). qPCR conditions included a heat activation step at 95°C for 15 min followed by 45 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 20 s. Actin qPCRs were applied to control for C6/36 replication (Henrichfreise et al., 2009). For actin qPCRs, a reaction mixture contained 1x HotStar Taq polymerase buffer, 1 mM $MgCl_2$, 200 µM dNTPs, 0.2 µL SYBR Green (1,000-fold diluted in DMSO), 0.3 µM actin primers (forward: 5'-ACGAACTGGGACGATATGGA-3′, reverse: 5'-GCCTCTGTCAGGAGAACTGG-3′; Microsynth, Balgach, Switzerland), 0.5 U HotStar Taq polymerase and 2 µL of extracted gDNA (1:20 diluted in AE buffer for cell culture samples, undiluted for cell-free samples). qPCR conditions included a heat activation step at 95°C for 15 min followed by 45 cycles of 95°C for 10 s, 57°C for 15 s, and 72°C for 20 s. Melt curve analysis showed a specific peak for all positive samples. Data were analyzed using Rotor-Gene 6,000 software version 1.7 (Corbett Life Sciences, Sydney, Australia). The fold change in 16S rRNA gene and actin copies is calculated by dividing the value of each time point by the mean copy number at D0 and indicates replication of *Wolbachia* and C6/36 cells, respectively.

2.8 Fluorescence microscopy of antibiotic-treated cell-free *Wolbachia*

Cell-free *Wolbachia* cultures were prepared as described above with 0.5×10^3 16S rRNA gene copies/µL isolated *Wolbachia* and Fraction 1 from insect cell lysate equivalent to 0.95×10^6 cells/mL diluted in standard medium with 20% FBS, cultured in 96-well plates at 26°C for 12 days and treated with 512 µg/mL fosfomycin (Infectofos, InfectoPharm, Heppenheim, Germany) daily or every three days with ampicillin (Sigma-Aldrich), bacitracin (AppliChem, Darmstadt, Germany), or vancomycin (Sigma-Aldrich). 50 µL of cell-free *Wolbachia* were dried on a microscopy slide and stained as described for the infection experiment. Cell diameter of fosfomycin-treated cells was measured based on the *w*Pal staining using ImageJ (Version 2.0.0-rc-43/1.50e, https://imagej.nih.gov/ij/).

2.9 Statistical analysis

For statistical analysis, GraphPad Prism version 10.1.2 for Windows (GraphPad Software, Boston, Massachusetts United States, www.graphpad.com) was used.

3 Results

3.1 *Wolbachia* replicate under cell-free conditions

As a first step toward establishing an insect cell-free culture of replicating *Wolbachia*, it was investigated whether lysate from disrupted host cells is sufficient for wolbachial growth. For this, two different cell-free *Wolbachia* suspensions were prepared. The first suspension contained *Wolbachia* purified according to the procedure published by Rasgon et al. (2006). The second suspension contained *Wolbachia* purified according to an abbreviated protocol in which the high-speed centrifugation on a sucrose cushion and the subsequent filtration step through a 1.2 µm filter were omitted, which retained more of the insect cell lysate. A 1:5 dilution of each suspension in standard medium was incubated in 25 cm^2 cell culture flasks at 26°C for up to 15 days. Samples were removed every one to three days (exact timing is shown in figures) and the number of *Wolbachia* was determined by qPCR. Gene copy numbers were normalized to the counts on day 0 of the culture. In the cell-free *Wolbachia* culture with retained insect cell lysate, 16S rRNA gene copies increased 3.2-fold by day 5 and 13-fold by day 13 (Figure 1). In contrast, the number of cell-free *Wolbachia*, purified as described by Rasgon et al. (2006) and thus without insect cell lysate, decreased by 88% from day 0 to day 3 and remained unchanged until day 9. An apparent increase was observed on day 11; however, considering the absence of subsequent replication and this time point being a single replicate, this data point was considered an outlier. *Actin* copy numbers were monitored to exclude the possibility that intact C6/36 cells remained in the culture; no increase in *actin* copy number was measured (Supplementary Figure S1). In the following, *Wolbachia* were purified using the abbreviated protocol.

We wanted to further characterize the conditions for cell-free growth of *Wolbachia* to enable consistent assays. In addition, faster growth of cell-free *Wolbachia* would be desirable to allow easy application, e.g., for antibiotic assays. Since the starting amount of C6/36 cells was not measured, our next step was to first determine the optimal amount of insect cell lysate.

3.2 *Wolbachia* replication is inversely dependent on the amount of lysate from uninfected C6/36 cells

Purified *Wolbachia* ($0.5–1.5 \times 10^3$ 16S rRNA gene copies/µL) were incubated with different dilutions of total cell lysate prepared from uninfected C6/36 cells. *Wolbachia* replication was detected in all dilutions of cell lysate, with the highest overall copy number of the 16S rRNA gene on day 9 (Figure 2). In lysate equivalent to 3.8×10^6 cells/mL, *Wolbachia* numbers increased up to 1.9-fold compared to day 0. In more diluted insect cell lysates, the *Wolbachia* replication rate was even higher, achieving an up to 2.9-fold increase with lysate from 1.9×10^6 cells/mL and up to 5.1-fold with lysate from 0.95×10^6 cells/mL. *Wolbachia* growth was achieved from day 0 to day 9 when using the two higher-concentrated lysates, whereas lower *Wolbachia* concentrations were measured on day 12. For the lowest lysate concentration, growth was also observed to day 12. Based on these results, cell lysate prepared from uninfected C6/36 cells equivalent to 0.95×10^6 cells/mL was used for further experiments.

3.3 Replication in cell-free medium is *Wolbachia* density-dependent

Next, the optimal initial density of *Wolbachia* for growth in cell-free culture was titrated. *Wolbachia* were purified from infected C6/36 cells and total cell lysate from uninfected C6/36 cells was prepared.

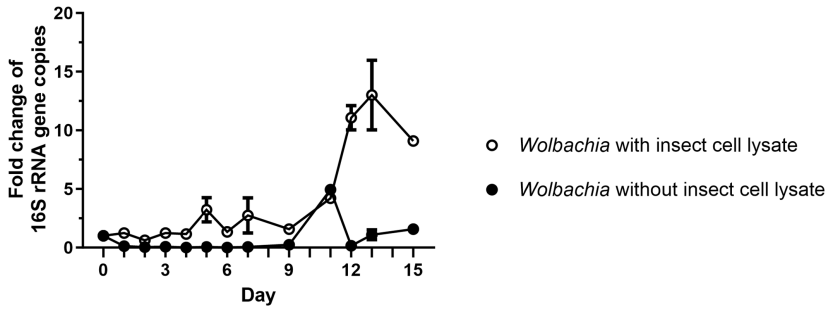

FIGURE 1
Isolated *Wolbachia* replicate in medium when the C6/36 cell membranes are retained. *Wolbachia* were purified from C6/36 cells via ultracentrifugation (Rasgon et al., 2006), or were purified by an abbreviated protocol that retained more of the insect cell lysate. Cell-free cultures were incubated at 26°C for 15 days and samples were taken every one to three days. *Wolbachia* were quantified by qPCR of the 16S rRNA gene. Copy numbers were normalized to day 0. Data were pooled from two independent experiments. For days 2, 4, 6 (experiment 1) and days 9, 11, 15 (experiment 2), the data from only one experiment is shown. For the other days, the mean ± SEM of 2–5 wells is shown.

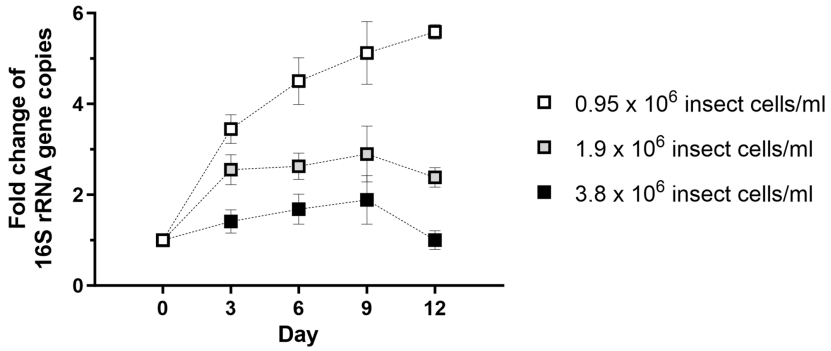

FIGURE 2
Wolbachia replication in cell-free culture is dose-dependent on the amount of C6/36 cell lysate. Total cell lysate from uninfected C6/36 cells was prepared from the depicted cell numbers determined in a Neubauer counting chamber prior to cell lysis. Purified *Wolbachia* ($0.5–1.5 \times 10^3$ 16S rRNA gene copies/µL) were incubated at 26°C for 12 days with the three indicated dilutions of insect cell lysate. Growth was monitored by 16S rRNA gene qPCR every three days and data were normalized to day 0. Data were pooled from two independent experiments. For every time point, the mean ± SEM of six wells is shown.

Decreasing concentrations of *Wolbachia* from 10^5 to 10^2 16S rRNA gene copies/µL were suspended in standard medium containing total insect cell lysate. In cell-free culture containing high *Wolbachia* counts of 10^5 or 10^4 16S rRNA gene copies/µL, the counts slightly increased 1.6- and 1.9-fold, respectively, until day 9 (Figure 3). In contrast, cultures containing 10^3 or 10^2 16S rRNA gene copies/µL had higher replication rates between days 0 and 9, increasing 3.6- and 4.7-fold, respectively. At all concentrations, *Wolbachia* numbers decreased to day 12. Therefore, unless otherwise stated, initial *Wolbachia* concentrations between 10^2 and 10^3 16S rRNA gene copies/µL were used for further experiments.

3.4 Insect cell membranes are essential for *Wolbachia* replication

Wolbachia replication might be dependent on soluble signaling molecules or growth factors provided by the C6/36 cells. As a first step to verify this possibility, insect cell lysate was separated by centrifugation to achieve a rough fractionation of C6/36 cell components (Lodish et al., 2000). For this, insect cell lysate was centrifuged at 20,000 g for 30 min or ultracentrifuged at 100,000 g for 60 min. The supernatant after 20,000 g centrifugation containing cytosol, microsomes, and plasma membranes of the C6/36 cells was retained (Fraction 1), and the corresponding pellet containing nuclear debris and large cell organelles was resuspended in standard medium (Fraction 2). The supernatant after ultracentrifugation containing soluble cytoplasmic contents was also retained (Fraction 3). All three fractions equivalent to 0.95×10^6 cells/mL were incubated separately with 10^3 16S rRNA gene copies/µL of purified *Wolbachia*. As controls, *Wolbachia* were grown in total insect cell lysate and in standard medium without lysate.

Wolbachia incubated with Fraction 1 had equivalent replication as *Wolbachia* incubated with total insect cell lysate, reaching 7-fold mean replication on day 9 compared to day 0. However, the group with Fraction 1 showed growth until day 12 (Figure 4). *Wolbachia*

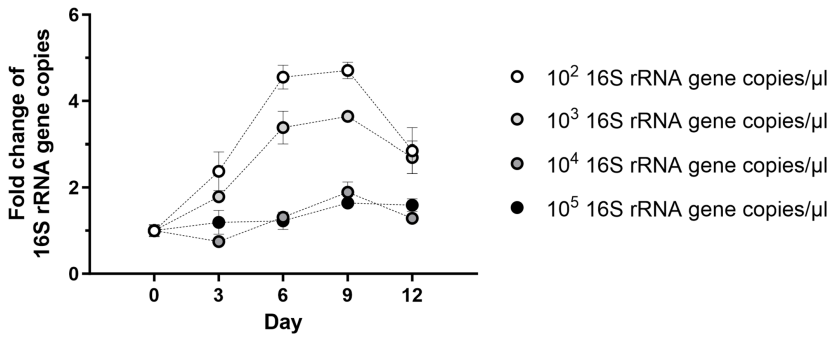

FIGURE 3
Starting density of *Wolbachia* influences cell-free replication. Different starting concentrations of *Wolbachia* were incubated with total insect cell lysate (equivalent to 0.95 × 10^6 uninfected C6/36 cells) at 26°C for 12 days. Growth was monitored by 16S rRNA gene qPCR every three days and data were normalized to day 0. The graph is representative of two independent experiments. For every time point, the mean ± SEM of three wells is shown.

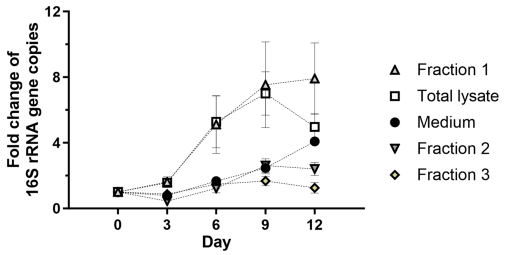

FIGURE 4
The cell membrane-containing fraction from C6/36 cells is required for *Wolbachia* replication in a cell-free culture. Total cell lysate was prepared from 0.95 × 10^6 uninfected C6/36 cells. A portion of cell lysate was fractionated by centrifugation at 20,000 g for 30 min or 100,000 g for 60 min. *Wolbachia* were incubated in the supernatant retained after 20,000 g centrifugation (Fraction 1, microsomes and membranes), the corresponding pellet resuspended in cell culture medium (Fraction 2, nuclear debris and organelles), or the supernatant retained after 100,000 g (Fraction 3, soluble cytoplasmic molecules) at 26°C for 12 days. Growth was compared to reactions containing total insect cell lysate or medium alone. The initial concentration of *Wolbachia* was 10^3 16S rRNA gene copies/µL. Growth was monitored by 16S rRNA gene qPCR every three days and data were normalized to day 0. Data were pooled from two independent experiments. For every time point, the mean ± SEM of six wells is shown, except for the medium group for which the mean ± SEM of three wells is shown.

incubated in medium alone or supplemented with Fraction 2 or Fraction 3 had similar growth curves until day 9 when they had replicated 2- to 3-fold. The medium group continued to replicate until day 12, never reaching more than 50% growth compared to the culture with Fraction 1, while the other two had a slight decrease. To investigate whether Fraction 2 or 3 contain compounds with an inhibitory effect on extracellular wolbachial growth, combinations of all fractions were tested. Replication rates were lower for Fraction 1 in combination with Fraction 2 or Fraction 3 than for Fraction 1 alone, indicating inhibitory effects (Supplementary Figure S2). Therefore, for all further experiments, cell-free *Wolbachia* cultures were supplemented with Fraction 1 (= membrane fraction).

To further extend cell-free growth, freshly prepared Fraction 1 was applied to the culture on day 9 and replication was monitored via qPCR until day 15. Growth rates were slightly higher in the supplemented group on day 12 (5-fold) than in the standard cell-free culture (4-fold), but growth was not prolonged since both groups showed a decrease to day 15 (Figure 5A). A second supplementation with fresh Fraction 1 on day 12 also did not extend cell-free *Wolbachia* replication (data not shown). Insufficient amounts of cholesterol were also considered to be a potential limiting factor of cell-free *Wolbachia* growth. Thus, freshly prepared water-soluble cholesterol was added to the cell-free culture with Fraction 1, but no increase in replication was observed (Figure 5B).

3.5 FBS is essential for *Wolbachia* replication

The growth rate of C6/36 insect cells in cell culture medium is slower in FBS-free medium (Kuno, 1983). The exact components of FBS are not known but many hormones, growth factors, and nutrients are provided with the serum. Thus, it was investigated whether FBS also supports or is necessary for *Wolbachia* replication in the cell-free system. *Wolbachia* grown in standard medium supplemented with Fraction 1 replicated as seen before, reaching a 4.2-fold increase on day 12 (Figure 6). In contrast, *Wolbachia* incubated in medium barely replicated, with a 1.3-fold increase on day 12. No increase of cell-free *Wolbachia* was detected when grown in FBS-free medium supplemented with Fraction 1 derived from uninfected C6/36 cells also harvested in FBS-free medium. These results show that both FBS and Fraction 1 are necessary for replication of cell-free *Wolbachia*; one without the other is not sufficient.

3.6 *Wolbachia* from cell-free culture can infect C6/36 cells

Rasgon et al. (2006) and Nevalainen et al. (2023) demonstrated that purified *Wolbachia* can infect uninfected insect cells. Therefore, we wanted to determine if *Wolbachia* that replicated in our cell-free culture system had maintained the infective phenotype. The infectivity of *Wolbachia* was examined by infecting uninfected C6/36 cells with *Wolbachia* grown in cell-free culture for 12 days (Figure 7A). For cell-free cultured *Wolbachia* incubated with Fraction 1, the number of *Wolbachia* increased 2.7-fold to day 12 (Figure 7A). *Wolbachia* from day

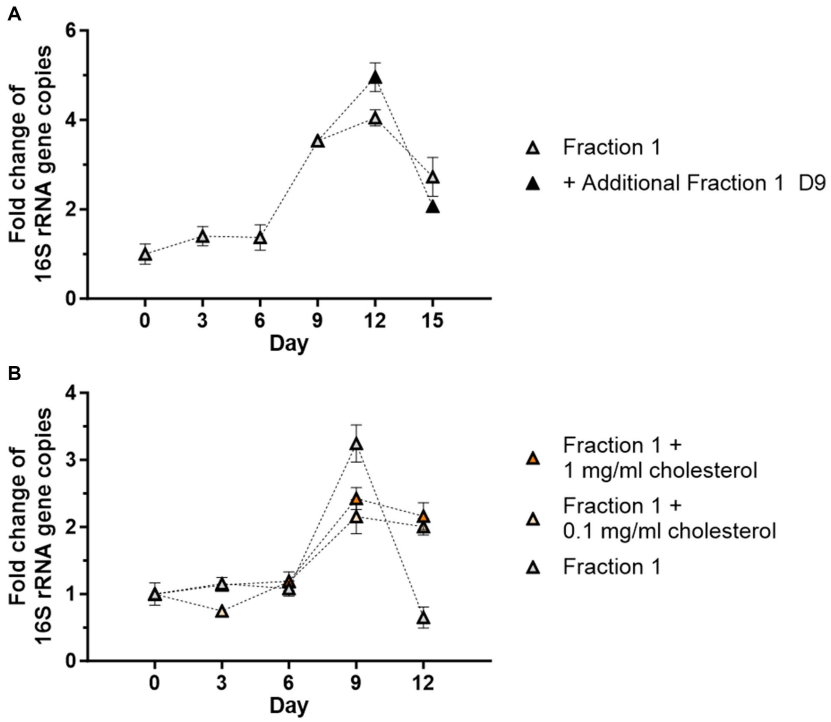

FIGURE 5
Addition of fresh Fraction 1 or cholesterol do not support cell-free replication. **(A)** Cell-free *Wolbachia* (2×10^2 16S rRNA gene copies/µL) were incubated with Fraction 1 from uninfected C6/36 cells (equivalent to 0.95×10^6 cells/mL) at 26°C for 15 days. On day 9, fresh Fraction 1 was added to half of the remaining wells. Growth was monitored by 16S rRNA gene qPCR every three days and data were normalized to day 0. The graph is representative of two independent experiments. For every time point, the mean ± SEM of three wells is shown. **(B)** Cell-free *Wolbachia* (0.5×10^3 16S rRNA gene copies/µL) were incubated with Fraction 1 from uninfected C6/36 cells (equivalent to 0.95×10^6 cells/mL) with or without water-soluble cholesterol (0.1 or 1 mg/mL) at 26°C for 12 days. Growth was monitored by 16S rRNA gene qPCR every three days and data were normalized to day 0. For every time point, the mean ± SEM of six wells is shown.

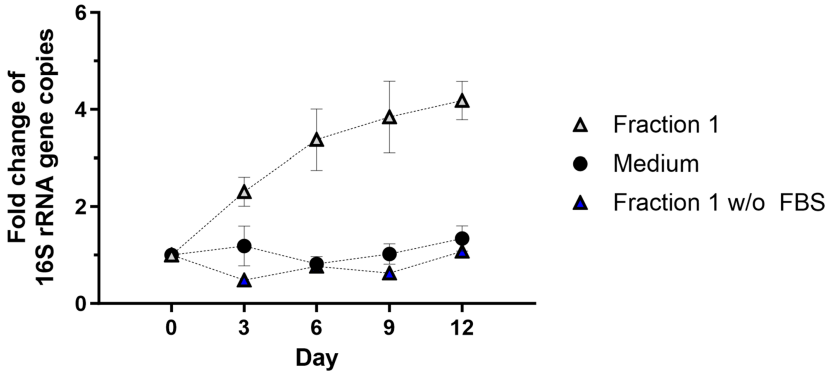

FIGURE 6
FBS is required for *Wolbachia* replication in a cell-free culture. Cell-free *Wolbachia* ($0.1–1 \times 10^4$ 16S rRNA gene copies/µL) were incubated with Fraction 1 from uninfected C6/36 cells (equivalent to 0.95×10^6 cells/mL) harvested in cell culture medium either with or without FBS and incubated at 26°C for 12 days. Growth was monitored by 16S rRNA gene qPCR every three days and data were normalized to day 0. Data were pooled from two independent experiments. For every time point, the mean ± SEM of six wells is shown.

12 of this cell-free culture were used to infect C6/36 cells. Six days post-infection, ~140 16S rRNA gene copies/µL were measured in the cell culture (Figure 7B). In contrast, only eight 16S rRNA gene copies/µL were detected in C6/36 cells infected with heat-killed *Wolbachia* from the same cell-free culture. Immunofluorescence microscopy using a *Wolbachia*-specific antiserum against *w*Pal confirmed the presence of

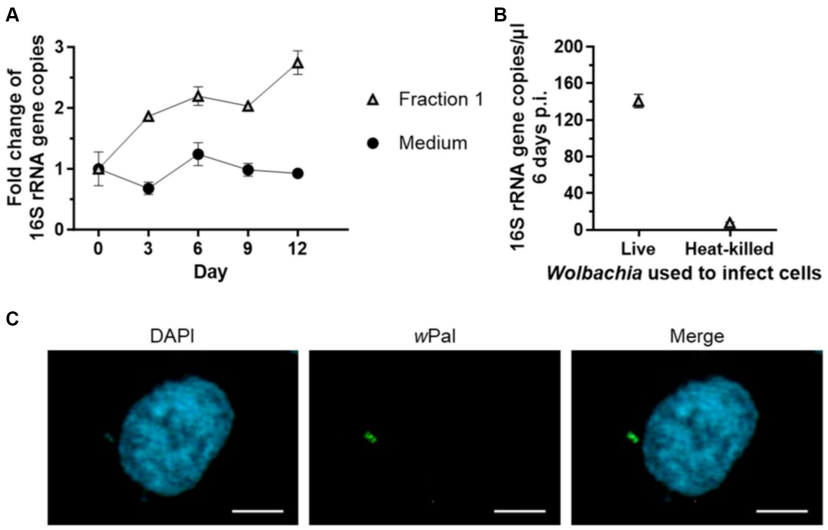

FIGURE 7
Cell-free cultured *Wolbachia* infect uninfected C6/36 cells. **(A)** Cell-free *Wolbachia* (0.5×10^3 16S rRNA gene copies/µL) were incubated with Fraction 1 from uninfected C6/36 cells (equivalent to 0.95×10^6 cells/mL) at 26°C for 12 days. Growth was monitored by 16S rRNA gene qPCR every three days. For every time point, the mean ± SEM of three wells is shown. **(B)** On day 12, 750 µL of this cell-free *Wolbachia* culture were added to uninfected C6/36 cells grown in a 24-well plate. As a negative control, *Wolbachia* were heat-killed at 95°C for 10 min prior to addition to the uninfected C6/36 cells. After centrifugation at 2,000 g for 1 h at 15°C, the plate was incubated overnight at 26°C. On the next day, the medium was removed and fresh cell culture medium was added. On day 6 post-infection, three samples were taken for 16S rRNA gene qPCR of C6/36 cells infected with *Wolbachia* and with heat-killed *Wolbachia* (mean ± SEM). Data are representative of two experiments. **(C)** Six days post-infection, C6/36 cells were grown on culture slides for 1 day and subsequently examined with a Zeiss Axio Observer.Z1 fluorescence microscope using immunofluorescence microscopy with *w*PAL anti-serum and an Alexa 488-conjugated secondary antibody (green, *Wolbachia*) and counterstained with DAPI (blue). Scale bar: 5 µm.

Wolbachia in the C6/36 cell culture 7 days post-infection when infected with live *Wolbachia* (Figure 7C).

3.7 *Wolbachia* replication rate is lower in cell-free culture

By supplementing standard medium with Fraction 1, *Wolbachia* were able to replicate for at least 9 days. To determine the stability and growth efficiency of *Wolbachia* in the cell-free culture system, growth rates of *Wolbachia* cultured with and without Fraction 1 were compared to *Wolbachia* cultured within the C6/36 cell line. For each culture system, the fold increase of 16S rRNA gene copies on day 9 was compared. Growth rates significantly differed between the groups (Figure 8). *Wolbachia* residing in C6/36 cells had a median growth rate of 14.8 (range 4.8–25.5; mean: 13.6). Cell-free *Wolbachia* cultured in standard medium had a median growth rate of 1.0 (range 0.6–6.8; mean: 1.7). Cell-free *Wolbachia* cultured in standard medium supplemented with Fraction 1 had a median growth rate of 4.2 (range 2–14.8; mean: 6.4).

3.8 Cell-free cultured *Wolbachia* are sensitive to fosfomycin treatment

It has been shown that *Wolbachia* are sensitive to fosfomycin (Henrichfreise et al., 2009), a specific inhibitor of MurA that catalyzes the first dedicated step of lipid II biosynthesis. Treatment of

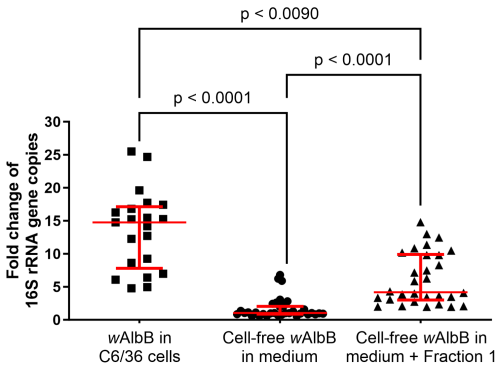

FIGURE 8
Variation in growth between *Wolbachia* cultured in cell-free medium ± Fraction 1 from C6/36 cell lysate compared to standard C6/36 cell culture. The replication of *Wolbachia* in cell-free culture with and without Fraction 1 from insect cell lysate or in C6/36 cells was compared on day 9, combining data from at least 20 independent experiments performed in duplicates (growth in C6/36 cells) or triplicates (cell-free growth), respectively. Cell-free *Wolbachia* with initial concentrations of 10^2–10^3 16S rRNA gene copies/µL were incubated with Fraction 1 from uninfected C6/36 cells (equivalent to 0.95×10^6 cells/mL). *Wolbachia* in C6/36 cells had initial concentrations of 10^3–10^4 16S rRNA gene copies/µL. Each dot represents one experiment. The median with interquartile range is shown (red lines). Statistical differences were determined using a Kruskal-Wallis test followed by a post-hoc Dunn's multiple comparisons test using GraphPad Prism 10.

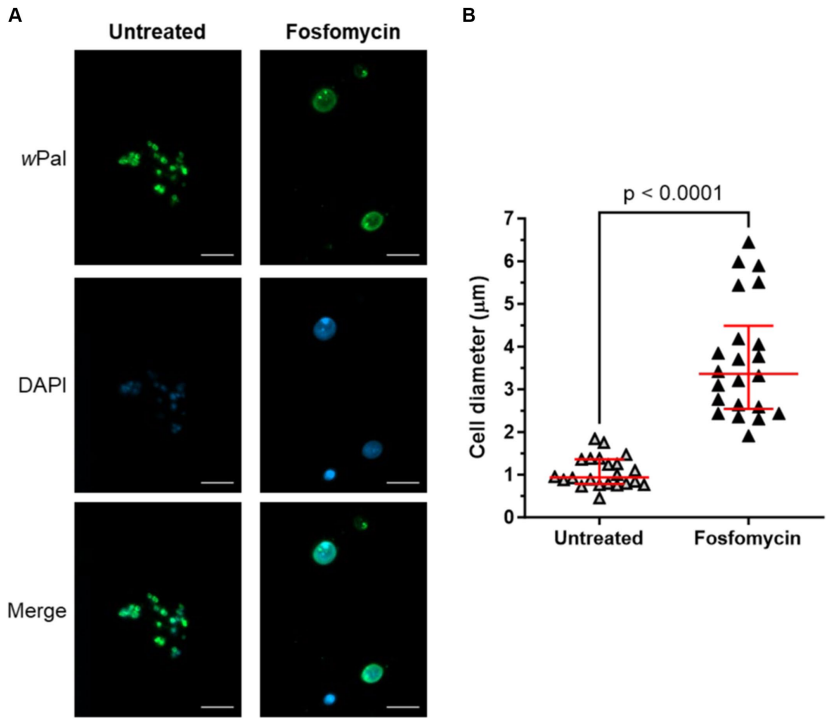

FIGURE 9
Cell-free cultured *Wolbachia* are sensitive to fosfomycin treatment. Cell-free *Wolbachia* (0.5×10^5 16S rRNA gene copies/µL) were incubated with Fraction 1 from uninfected C6/36 cells (equivalent to 0.95×10^6 cells/mL) at 26°C for 12 days, with or without daily 512 µg/mL fosfomycin treatment. **(A)** Cells were fixed and visualized by immunofluorescence microscopy using *w*PAL anti-serum and an Alexa 488-conjugated secondary antibody (green, *Wolbachia*) and counterstained with DAPI (blue). Scale bar: 5 µm. **(B)** Cell diameter (median with IQR, red lines) was measured with ImageJ based on the *w*Pal staining from three independent assays ($n = 22$). Statistical differences were determined using a Mann–Whitney test using GraphPad Prism 10.

Wolbachia-infected C6/36 cells with fosfomycin resulted in fewer and enlarged *Wolbachia* cells, demonstrating that the cell wall precursor lipid II is necessary for cell division in *Wolbachia* (Vollmer et al., 2013). To confirm that cell-free cultivated *Wolbachia* are suitable for antibiotic studies, e.g., to understand the reduced cell division machinery encoded in the genome, the phenotype of fosfomycin-treated endobacteria was analyzed via immunofluorescence microscopy using anti-*w*PAL. Untreated cell-free *Wolbachia* had a median (IQR) cell diameter of 0.94 µm (0.78–1.36 µm), whereas fosfomycin-treated *Wolbachia* were significantly larger with 3.36 µm (2.54–4.49 µm) (Figure 9). We only observed these enlarged cells when we also detected wolbachial replication via qPCR of the 16S rRNA gene. In contrast, the other cell wall biosynthesis-inhibiting antibiotics tested, i.e., ampicillin, bacitracin, and vancomycin did not affect the phenotype of cell-free *Wolbachia* (Supplementary Figure S3).

4 Discussion

In vitro culture systems of *Wolbachia* necessary for the elucidation of their biology are few and only *Wolbachia* strains naturally occurring in arthropods have been successfully cultured in insect cell lines (Fenollar et al., 2003a; McMeniman et al., 2008), while all attempts to culture *Wolbachia* of filarial nematodes have failed (McNulty et al., 2010; Slatko et al., 2014; Marriott et al., 2023). Molecular biological techniques are mostly impossible to apply to *Wolbachia* cultured in insect cell lines, e.g., genetic transformation or treatment of *Wolbachia* with large antibiotics such as aminoglycosides, polymyxins, lipo-and glycopeptide antibiotics that might not pass the insect cell membranes. Therefore, the extracellular cultivation of *Wolbachia* would provide an excellent tool for understanding the biology and symbiosis of *Wolbachia*. However, *Wolbachia* purified from insect cells have only been maintained without replication in cell-free cultures (Rasgon et al., 2006; Krafsur et al., 2020). Further attempts regarding *ex vivo* growth failed, but some components were advantageous regarding survival of *Wolbachia*, e.g., compatible solutes, actin, and mammalian blood (Uribe-Alvarez et al., 2018).

For other intracellular species such as *Coxiella burnetii* a complex medium has been designed in which cell-free growth occurred (Omsland et al., 2009). However, contrary to *Wolbachia*, *Coxiella burnetii* exhibit a less symbiotic interaction with their host cell and can even persist in an extracellular environment (Heinzen et al., 1999). Furthermore, attempts to generate a complex medium for cell-free growth of *Chlamydia*, which have a lifestyle that is more similar to that of *Wolbachia*, were unsuccessful (Omsland et al., 2012). This points out the complexity of cell-free growth of obligate intracellular bacteria that are tightly associated with their host. Compared to *Coxiella burnetii*, *Wolbachia* and *Chlamydia* possess a

substantially reduced genome, which might make cell-free growth even more difficult.

In the present study, we could demonstrate that *Wolbachia* were not only viable when maintained in a cell-free culture, but underwent replication when insect cell lysate from uninfected C6/36 cells was added to the medium. In some experiments, a slight increase in *Wolbachia* numbers was observed in standard cell culture medium without insect cell lysate, but it never reached the levels observed when supplementing the cell-free medium, and in most cases no growth was detected. It is possible that the *Wolbachia* suspension generated from infected C6/36 cells contained sufficient components that allowed for a weak replication rate.

Viability and infectivity of *Wolbachia* from a 12-day-old cell-free *Wolbachia* culture were confirmed by infecting uninfected C6/36 cells, with *Wolbachia* DNA and intracellular *Wolbachia* detectable six and seven days post-infection. With a maximum of 1–2 *Wolbachia* per C6/36 cell, the insect cells were considerably less infected than the Aa23 and JW18 cells of Rasgon et al. (2006) and Nevalainen et al. (2023), respectively. While this could be supported by different uptake efficiencies of the *Wolbachia* subspecies and insect cells, it is most likely explained by the different MOIs used for infection. We used an MOI of 14, whereas Rasgon et al. (2006) used an MOI of 2,600 and Nevalainen et al. (2023) used an MOI of 20:1 host cell equivalents (i.e., the *Wolbachia* contents from 20 infected cells per seeded uninfected cell). Our MOI was low because of the low *Wolbachia* numbers required for cell-free replication. We also used a comparatively high number of C6/36 cells to have confluent growth and thereby increase the likelihood of *Wolbachia* coming into contact with a C6/36 cell. When *Wolbachia* were killed by heating prior to infection of uninfected C6/36 cells, only minimal amounts of DNA were detected in the cells 6 days post-infection, confirming the results from Nevalainen et al. (2023) that *Wolbachia* are not only passively taken up but also facilitate their uptake. No *Wolbachia* could be detected by immunofluorescence microscopy (data not shown). Thus, detected DNA most probably represents residual DNA from dead *Wolbachia*.

Wolbachia growth in the insect cell-free culture was dependent on the initial *Wolbachia* concentrations, with higher concentrations resulting in lower levels of replication. A first explanation might be an insufficient supply of nutrients, e.g., pyruvate and intermediates of the tricarboxylic acid cycle derived from amino acids (Foster et al., 2005). However, 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 competition for nutrients is unlikely. Instead, *Wolbachia* densities might be regulated by a yet unknown, intrinsic, or host cell-derived mechanism. It is striking that cell-free *w*AlbB showed the highest replication rates at an initial concentration of $0.1–1 \times 10^3$ 16S rRNA gene copies/µL. In contrast, in cell-free cultures containing higher densities of *Wolbachia* with 10^4 or 10^5 16S rRNA gene copies/µL, *Wolbachia* numbers only slightly increased. This indicates that *Wolbachia* might sense densities and regulate cell division by internal communication patterns. The two-component regulatory system (TCS) is the predominant form of signaling used in a majority of prokaryotes, including bacteria (Beier and Gross, 2006). It is composed of a sensor histidine kinase and a paired response regulator (Mitrophanov and Groisman, 2008; Jung et al., 2012). Stimuli such as nutrients, osmolarity, oxygen, salinity, and quorum sensing cues are recognized by sensor histidine kinases (Mascher et al., 2006). This activates cognate response regulators which, e.g., coordinate induction of sporulation, regulation of bacterial differentiation, or formation of biofilms (Stock et al., 2000). TCS genes are highly conserved in various *Wolbachia* strains, but very little is known about their function to date (Cheng et al., 2006; Brilli et al., 2010). A bioinformatics study showed that wolbachial TCS genes are consistently found clustered with metabolic genes within several *Wolbachia* strains, including *w*AlbB and *w*Bm (Christensen and Serbus, 2015). Considering these findings, it might be hypothesized that *Wolbachia* are able to sense, e.g., nutrients or quorum sensing molecules and consequently regulate cell division and density. This could explain why cell-free *Wolbachia* growth stops after 9–12 days of incubation and could further explain the observation that *Wolbachia* cell numbers inside C6/36 cells do not reach a density that would negatively affect the survival of their host cell. Nevertheless, how *Wolbachia* growth is regulated remains to be elucidated.

It was also observed that increasing the amount of uninfected C6/36 cells used to prepare total insect cell lysate had a detrimental effect on *Wolbachia* growth rather than increasing replication. *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 cell culture was originally generated from *A. albopictus* larvae and therefore consists of cells of different cell cycle stages and of different cell types (Singh, 1967; Igarashi, 1978). Hence, it should be considered that *Wolbachia* growth-inhibiting factors present in a subset of C6/36 cells might accumulate when larger numbers of cells are used for lysate preparation. Fraction 1 (containing microsomes and plasma membranes) induced *Wolbachia* growth. However, almost no replication occurred when Fraction 2 (nuclear debris and large organelles) or Fraction 3 (soluble cytoplasmic content) of the C6/36 cells were used to supplement the medium. Since a combination of Fraction 1 with either Fraction 2 or Fraction 3 decreased growth, we hypothesize an inhibiting effect of these fractions. Supplementation with fresh Fraction 1 on day 9 enhanced growth to day 12 but could not extend growth. A second supplementation with fresh Fraction 1 on day 12 also failed to prolong growth. The factor limiting cell-free growth to 12 days remains unclear. As the fresh Fraction 1 was only added to existing cultures and the medium was not completely exchanged, there might be degradation products present that prevented further growth of the cell-free *Wolbachia*.

Notably, it has been shown that survival of endobacteria of the species *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*, which are closely related to *Wolbachia* spp., is dependent on the incorporation of cholesterol derived from their host cell (Lin and Rikihisa, 2003). Like *Wolbachia*, *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* do not synthesize lipid A and it was proposed that cholesterol might be necessary to promote membrane stability as a substitute for lipopolysaccharides (Lin and Rikihisa, 2003; Wu et al., 2004). There are indications that *Wolbachia*-infected insect cells might indeed incorporate cholesterol (Caragata et al., 2013; Geoghegan et al., 2017). Further, *Wolbachia* reside in cholesterol-rich Golgi-related vesicles derived from the host which form a vacuole surrounding each bacterium (Cho et al., 2011). Insects assimilate cholesterol from their environment which is incorporated into the plasma membrane and internal membranes such as those from the Golgi apparatus (Rolls et al., 1997). Thus, cholesterol might be a limiting factor for cell-free *w*AlbB replication, and supplementation with the membrane fraction

of an insect cell lysate might not be sufficient to sustain growth for more than 12 days. However, the supplementation of water-soluble cholesterol did not lead to increased cell numbers under the conditions tested, indicating that this compound cannot be the only potential limiting growth factor. Apart from cholesterol, eukaryotic sphingomyelin was found in membranes of *Chlamydia trachomatis* (Carabeo et al., 2003), and it was shown that *Chlamydia* need these host lipids for expansion and replication (Feldkamp et al., 2017). Insects do not have sphingomyelin but instead contain ceramide phosphorylethanolamine (Luukkonen et al., 1973). Therefore, the sphingolipids sphingomyelin or ceramide phosphorylethanolamine, respectively, might be taken up by *Wolbachia* residing in different hosts and be essential for replication.

Nevertheless, components of Fraction 1 such as cholesterol cannot be the only necessary factor for *Wolbachia* growth outside their host cell since *Wolbachia* were not able to grow in cell-free medium supplemented with Fraction 1 derived from C6/36 cells harvested in medium without FBS. The composition of FBS is unknown but it is very likely that the serum, similar to the eukaryotic host cells in cell culture, provides proteins, carbohydrates, lipids, vitamins, and other factors essential for *Wolbachia* viability and replication. Similarly, extracellular growth of *Coxiella burnetii* was initially found to be FBS-dependent as well (Omsland et al., 2009), although a defined medium without FBS was developed later (Sandoz et al., 2016).

A prerequisite for bacterial cell division is the proper assembly of the divisome and disturbance of this process results in an aberrant phenotype characterized by swelling or filamentation of bacteria (Goehring et al., 2005; Park et al., 2005). For *Wolbachia* cultured in C6/36 cells, enlarged cells were observed subsequent to the blockade of lipid II biosynthesis by fosfomycin, demonstrating that the cell wall precursor lipid II is essential for the cell division of *Wolbachia* (Vollmer et al., 2013). A similar phenotype was induced by fosfomycin in intracellular *Protochlamydia* and *Waddlia chondrophila* (Pilhofer et al., 2013; Scherler et al., 2020). In the cell-free *Wolbachia* culture, the same aberrant phenotype was observed, indicating that the bacteria are indeed replicating in the cell-free system and that replication can be inhibited by fosfomycin. This was underlined by the fact that we only detected enlarged *Wolbachia* when we measured an increase of 16S rRNA gene copies via qPCR. The fosfomycin-treated cell-free *Wolbachia* were significantly enlarged with 3.36 µm (2.54–4.49 µm) [median (IQR)]. The determined cell diameter of 0.94 µm (0.78–1.36 µm) of the untreated cell-free *Wolbachia* fits well with the 0.8–1.5 µm determined by Hertig, showing that the cell-free *Wolbachia* display their normal morphology (Hertig, 1936).

We hypothesized a phenotype similar to the fosfomycin-induced for other cell wall biosynthesis-inhibiting antibiotics and thus tested ampicillin, bacitracin, and vancomycin. Belonging to the class of beta-lactam antibiotics, ampicillin binds to penicillin-binding proteins (Suginaka et al., 1972; Tipper, 1979). Since bacitracin and vancomycin are large antibiotics that might not be taken up by the C6/36 cells, we were interested in a possible effect on cell-free *Wolbachia*. Bacitracin binds to the pyrophosphate moiety of undecaprenyl pyrophosphate (C55-PP) and vancomycin binds to the D-Ala-D-Ala of lipid II (Perkins, 1969; Storm and Strominger, 1973). For all three, no effect on the phenotype of cell-free *Wolbachia* was observed although their intracellular targets are present (Henrichfreise et al., 2009; Vollmer et al., 2013; Atwal et al., 2021). Possibly, bacitracin and vancomycin are not reaching their targets due to the outer membrane of *Wolbachia* (Nikaido, 1989). Beta-lactams have previously been found to not affect intracellular *Wolbachia w*AlbB in cell culture, the reason is unclear (Fenollar et al., 2003b; Fallon, 2018). In contrast, for *Chlamydia*, an aberrant phenotype is induced by beta-lactams (Matsumoto and Manire, 1970; Kramer and Gordon, 1971), and for *Waddlia chondrophila*, an aberrant phenotype is induced by both beta-lactams and vancomycin (Scherler et al., 2020). Further investigation is necessary to determine why these cell wall biosynthesis-inhibiting antibiotics do not have a similar effect for *Wolbachia*.

Although close attention was paid to using consistent conditions, the cell-free cultures often did not grow. We also observed a decrease in cell-free growth rates over time, which could be due to a new FBS batch (Liu et al., 2023). In the calculation of the median growth rate, only the assays in which the *Wolbachia* replicated were included. The variance of growth rates between independent experiments in cell-free culture containing Fraction 1 was similar to those of *Wolbachia* cultured inside C6/36 cells. In both culture systems, we observed growth variability occurring over time that might originate from variances of medium or cell culture passage. However, the median growth rate of *Wolbachia* in cell-free medium is ~3.5 times lower compared to *Wolbachia* cultured in C6/36 cells. This indicates that in addition to the need for Fraction 1 for cell-free *Wolbachia* cultivation, further constituents are needed.

Previous studies indicate that replication of *Wolbachia* is dependent on the stage of the host life cycle, tissue-specific control mechanism, and host cell replication (Min and Benzer, 1997; McGraw et al., 2002; Ruang-areerate et al., 2004; Landmann et al., 2012). These findings provide insight into the complexity of *Wolbachia* replication, which will in turn influence the cell-free cultivation of the bacteria. In the cell-free culture, the differences in cell types and cell cycle stages of the C6/36 cells used to generate the lysate, and thus Fraction 1, could therefore have a major effect on replication. Further elucidation of this culture system will be necessary to achieve greater and sustained *Wolbachia* growth outside their host cells and to gain insight into the multiple mechanisms that influence and regulate replication in the symbiosis.

Nevertheless, the establishment of this culture system represents a further step in the effort to cultivate *Wolbachia* extracellularly and might also provide important cues for the extracellular cultivation of other endobacteria that could not be cultivated *in vitro* yet. 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.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

LB: Data curation, Formal analysis, Funding acquisition, Visualization, Writing – original draft, Writing – review & editing, Investigation, Methodology. KM: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing,

Investigation, Methodology. JV: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition, Investigation, Methodology. CC: Data curation, Formal analysis, Writing – review & editing, Investigation. AS: Conceptualization, Writing – review & editing. AH: Conceptualization, Funding acquisition, Writing – review & editing, Supervision. KP: Conceptualization, Funding acquisition, Project administration, Writing – review & editing, Methodology, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1405287/full#supplementary-material

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The MraY Inhibitor Muraymycin D2 and Its Derivatives Induce Enlarged Cells in Obligate Intracellular *Chlamydia* and *Wolbachia* and Break the Persistence Phenotype in *Chlamydia*

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Abstract: Chlamydial infections and diseases caused by filarial nematodes are global health concerns. However, treatment presents challenges due to treatment failures potentially caused by persisting *Chlamydia* and long regimens against filarial infections accompanied by low compliance. A new treatment strategy could be the targeting of the reduced peptidoglycan structures involved in cell division in the obligate intracellular bacteria *Chlamydia* and *Wolbachia*, the latter being obligate endosymbionts supporting filarial development, growth, and survival. Here, cell culture experiments with *C. trachomatis* and *Wolbachia* showed that the nucleoside antibiotics muraymycin and carbacaprazamycin interfere with bacterial cell division and induce enlarged, aberrant cells resembling the penicillin-induced persistence phenotype in *Chlamydia*. Enzymatic inhibition experiments with purified *C. pneumoniae* MraY revealed that muraymycin derivatives abolish the synthesis of the peptidoglycan precursor lipid I. Comparative in silico analyses of chlamydial and wolbachial MraY with the corresponding well-characterized enzyme in *Aquifex aeolicus* revealed a high degree of conservation, providing evidence for a similar mode of inhibition. Muraymycin D2 treatment eradicated persisting non-dividing *C. trachomatis* cells from an established penicillin-induced persistent infection. This finding indicates that nucleoside antibiotics may have additional properties that can break bacterial persistence.

Keywords: intracellular bacteria; *Chlamydia*; *Wolbachia*; cell division; peptidoglycan; lipid II synthesis; muraymycin; MraY; persistence-breaking

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1. Introduction

Bacteria of the genera *Chlamydia* and *Wolbachia* are obligate intracellular bacteria of medical importance. *Chlamydia* spp. are pathogens that undergo a unique biphasic developmental cycle within human epithelial and endothelial cells, whereas *Wolbachia* spp. reside in filarial nematodes and can cause diseases in humans.

Chlamydia infect host cells as condensed and metabolically less active elementary bodies (EBs). After invagination into the host cell, the EBs differentiate into non-infectious, metabolically active reticulate bodies (RBs) and replicate in the chlamydial inclusion. They

asynchronously differentiate back into EBs and are released by host cell lysis or extrusion. One of the most relevant chlamydial pathogens is *C. trachomatis*. Serovars A-C cause ocular infections that are the leading cause of preventable blindness worldwide [1], while serovars D-K lead to ano-urogenital infections, and serovars L1-L3 cause lymphogranuloma venereum. The latter two are sexually transmitted infections. In 2019, 434,184 confirmed cases of genital chlamydial infection were recorded in member states of the European Union/European Economic Area [2]. Infections such as cervicitis in women and urethritis in men are often asymptomatic, but chronic or untreated chlamydial infections can have detrimental consequences for women, e.g., pelvic inflammatory disease, ectopic pregnancy, and miscarriage [3,4]. Currently, the WHO recommends doxycycline (DOX) or azithromycin (AZI) treatment for uncomplicated genital *Chlamydia* infections [5]. However, high rates of reoccurring chlamydial infections after treatment have been reported, ranging from a median of 13% in a meta-study to 31% for females in the USA [6–8]. About 8% of treatment failures cannot be attributed to reinfection by sexual partners [8,9].

Besides the possibility of reinfection from the gastro-intestinal tract [10], persistent *C. trachomatis* infection should be taken into consideration, as enlarged chlamydial cells could be observed in murine tissue under amoxicillin treatment [11] and in tissue from patients [6,12]. These enlarged and non-dividing cells, referred to as aberrant bodies (ABs), can be found in cell culture in the presence of stressors such as β-lactams and interferon-γ [13,14]. ABs are less metabolically active, persist within the host cell, and can re-enter the developmental cycle after the removal of the stressor [14,15]. Persisting chlamydial cells, including those induced by penicillin (PEN) treatment, are less susceptible to DOX and AZI in cell culture and in murine infection models [11,16–18], a phenomenon that may also contribute to treatment failure with these front-line antibiotics in patients.

Wolbachia spp. can be found in a range of arthropods and filarial nematodes [19–21]. In arthropods, these endobacteria are mostly facultative symbionts [22], whereas in the filarial nematodes *Wuchereria bancrofti*, *Brugia malayi*, *Brugia timori*, and *Onchocerca volvulus*, they are obligate endosymbionts that support filarial development and survival [23,24]. Filarial infections cause the neglected tropical diseases lymphatic filariasis, transmitted by mosquitos, and onchocerciasis (river blindness), transmitted by blackflies. Worldwide, an estimated 52 million people had lymphatic filariasis in 2018, and the disease predominantly occurs in the tropical countries of Africa and Asia [25]. Onchocerciasis, predominantly found in Africa and in foci in Yemen and Latin America, affects 21 million people, causing severe skin disease and vision loss [26]. The drugs diethylcarbamazine or ivermectin used to treat lymphatic filariasis and onchocerciasis mainly kill the larvae in the blood or skin but not the adult worms [26]. Because the nematodes are dependent on *Wolbachia* spp. for development and adult worm survival, DOX or rifampicin, which both deplete the endobacteria from the worms, can also be used, with the benefit that the targeting of *Wolbachia* spp. leads to the killing of adult worms and, therefore, does not require annual or biannual treatment for the lifetime of the adult worms as it is required for diethylcarbamazine and ivermectin [23,24,27]. Anti-wolbachial treatment is the only safe and effective adult worm-killing therapy [28], and new drugs without the contraindications for DOX and rifampicin are needed.

Treatment failure of chlamydial infections, the high prevalence of *C. trachomatis* and diseases caused by filarial nematodes, and long treatment regimens in combination with low compliance and contraindications for filarial infections stress the need for new antimicrobial treatment strategies against *Chlamydia* and *Wolbachia*. An important antibiotic target in many bacteria is the peptidoglycan (PGN) layer within the cell envelope. PGN is unique to bacteria and usually consists of linear glycan strands connected by cross-linked peptides that form an exoskeleton-like meshwork that protects bacteria from osmotic stress. Inside their intracellular niche, *Chlamydia* and *Wolbachia* are protected from osmotic challenges, and the reductive adaptation to their host is reflected by the loss of a canonical energy-cost-intensive PGN-based cell wall [29–31].

However, both endobacteria retained reduced, but functional, PGN biosynthesis pathways that have been implicated in cell division [32–34]. *Chlamydia* synthesize a transient PGN ring that is required for a unique cell division process [29,30,35,36]. The exact PGN structure in *Wolbachia* is unknown, but a growing body of research provides evidence for a reduced PGN-like structure that is also important for cell division [31–33].

Antibiotics targeting PGN biosynthesis in the obligate intracellular *Chlamydia* and *Wolbachia* need to traverse several membranes, and due to the reduced synthesis machinery and distinct functions and structure of PGN in both endobacteria, these antibiotics may not act in the same manner as it has been defined in free-living bacteria. PEN binds to penicillin-binding proteins (PBPs) and thereby interferes with the transpeptidation and remodeling of PGN, which is usually bactericidal in many bacteria via not fully understood cellular downstream effects. PEN acts bacteriostatically in *Chlamydia* and induces a reversible state of persistence by blocking cell division, which leads to the formation of enlarged ABs that persist within the host cell [14,37–39]. *Wolbachia*, on the other hand, are unaffected by PEN treatment [40] but are susceptible to fosfomycin [32], which lacks activity against *Chlamydia* due to intrinsic resistance [41]. Fosfomycin targets the first synthesis step of the PGN precursor lipid II [42]. We previously showed that fosfomycin treatment also induces the formation of enlarged *Wolbachia* cells [33], a phenotype similar to that seen for PEN treatment of *Chlamydia*.

A target of interest within the lipid II synthesis pathway is the transferase MraY, a member of the polyprenyl-phosphate *N*-acetyl hexosamine 1-phosphate transferase superfamily. Our previous work showed that both *Chlamydia* and *Wolbachia* harbor functionally conserved MraY and MurG homologs and are thus capable of synthesizing the PGN precursor lipid II [32]. At the inner membrane of these two organisms, the soluble precursor uridine diphosphate (UDP)-*N*-acetylmuramoyl(Mur*N*Ac)-pentapeptide is first linked to the membrane carrier undecaprenyl-phosphate (bactoprenol, C_{55}-P) by the integral membrane protein MraY, yielding the penultimate PGN precursor, lipid I. Subsequently, the glycosylation of lipid I with UDP-*N*-acetylglucosamine (Glc*N*Ac) by MurG leads to the formation of lipid II [32].

MraY homologs from different free-living bacteria, e.g., *Bacillus subtilis*, *Staphylococcus aureus*, and *Aquifex aeolicus*, were shown to be inhibited by naturally occurring nucleoside antibiotics of *Streptomyces* [43–46] that are comprised of different subclasses. Two of these are muraymycins and caprazamycins, which both contain a 6′-*N*-alkyl-5′-beta-*O*-aminoribosyl-C-glycyluridine structure and display high activity against several Gram-positive pathogens, e.g., methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus* strains, and *Pseudomonas aeruginosa* [45,47–49]. Nucleoside antibiotics such as muraymycin (MRY) D2 are competitive inhibitors for the natural substrate of MraY, UDP-Mur*N*Ac-pentapeptide [45,47,50], due to the structural similarity of UDP-Mur*N*Ac to the nucleotide moiety of muraymycins. In contrast, the peptidic moiety of MRY D2 is non-competitive. During inhibition, a conformational change of the active site enables the binding of MRY D2 to MraY from *Aquifex aeolicus* [50].

In this study, we aimed to understand the PGN synthesis machinery in *Chlamydia* and *Wolbachia* and to explore the antimicrobial activity of muraymycins against these distinct non-model bacteria that have both functionally conserved the target of nucleoside antibiotics. Using cell culture-based infection models, we showed that these endobacteria are susceptible to muraymycins. In both genera, the MraY inhibitors induced the formation of enlarged cells that are similar to the chlamydial PEN-induced persistence phenotype. In complementary enzymatic studies with purified chlamydial MraY, we verified the integral membrane protein as a target of muraymycin D2 in *Chlamydia*. Based on our in silico analysis, we propose a similar mechanism for the binding of MRY D2 and its derivatives to the target protein MraY in *Chlamydia* and *Wolbachia* as that described for *A. aeolicus* MraY. When applied to PEN-induced persistent chlamydial infection, MRY D2 had persistence-breaking properties and eradicated the enlarged non-dividing chlamydiae from the host

cells, indicating that the nucleoside antibiotics may have further effects in addition to the inhibition of MraY.

2. Results

2.1. Muraymycin D2 and its Derivatives Induce the Formation of Enlarged Cells in C. trachomatis upon Treatment at an Early Stage of the Chlamydial Developmental Cycle

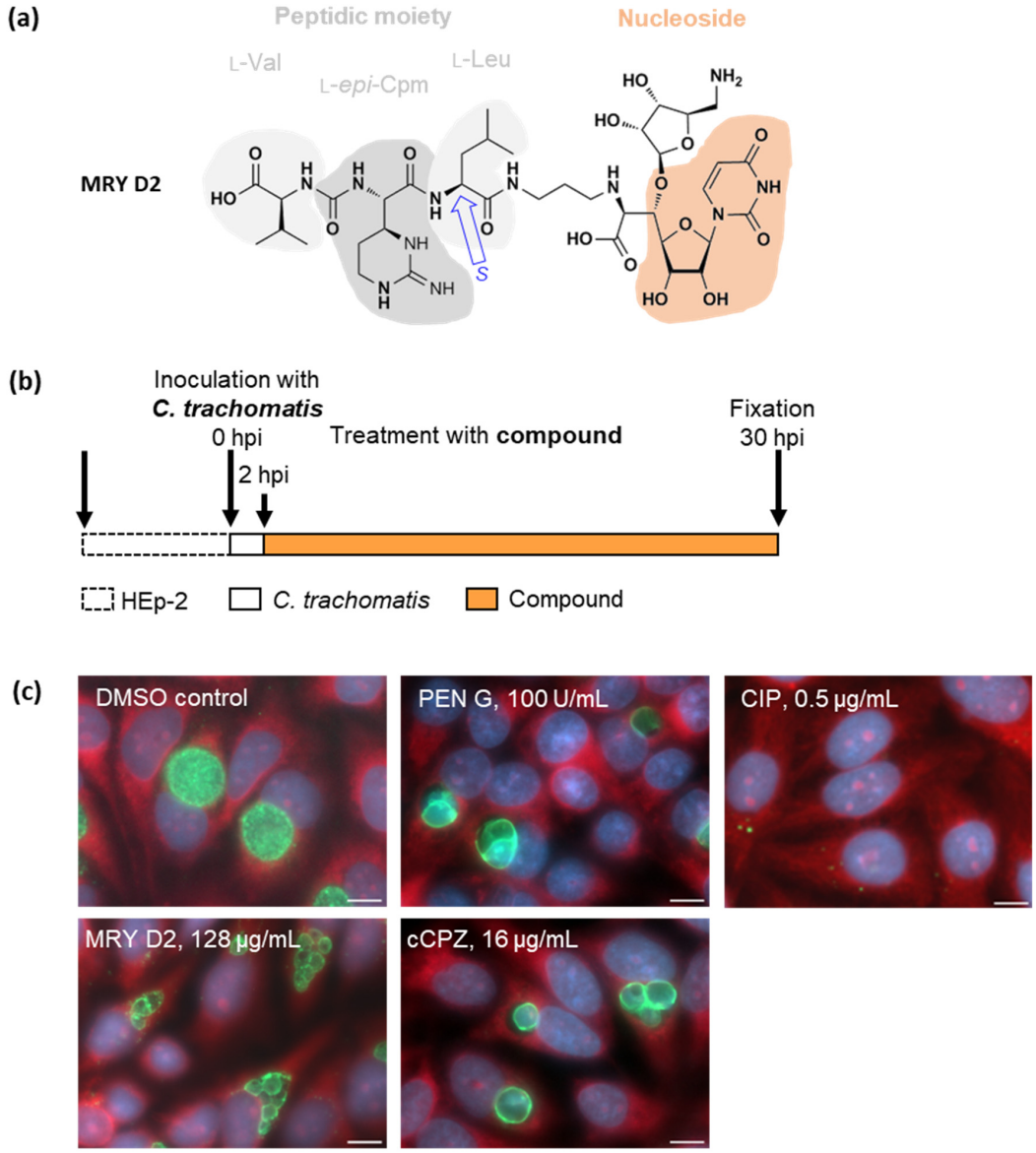
Treatment failure of genital chlamydial infections is not completely understood and needs to be addressed by understanding the underlying reasons and by searching for new antibiotics against the minimalist bacteria. Here, we explored the effects of muraymycins in obligate intracellular *Chlamydia*. These natural compounds block PGN synthesis through interference with the transferase MraY in free-living bacteria [45,47–50]. Since the enzyme is functionally conserved in *Chlamydia* [32], we postulated that muraymycins may have anti-chlamydial activity and hamper biosynthesis of the PGN ring that is essential in chlamydial cell division, as shown for the persistence-inducing antibiotic PEN.

First, we tested the lead compound MRY D2 (Figure 1a) and seven muraymycin derivatives (MRH; described in [47,48]) on *C. trachomatis* in cell culture experiments. To this end, *C. trachomatis* D/UW-3/CX-infected HEp-2 cells were treated with the compounds at 2 h post infection (hpi; Figure 1b), which represents an early stage of the chlamydial developmental cycle before bacterial replication [14,51]. Inhibitors were applied over a range of concentrations that were not detrimental to HEp-2 host cells in resazurin-based viability assays (Table S1), and the effects were visualized by fluorescence microscopy (Figures 1c and S2) at 30 hpi. The minimal inhibitory concentration (MIC) for *Chlamydia* was defined as either the lowest concentration leading to a >90% reduction in inclusions [52] or, for aberrance-inducing compounds, the concentration leading to abnormal chlamydial cell phenotypes, i.e., enlarged cells after treatment with PEN G [39].

As expected, our data showed that the PEN G control acted bacteriostatically, resulting in the presence of enlarged, non-dividing bacterial cells in the inclusion, whereas the ciprofloxacin control killed the pathogens and cleared the infection from the host cells (Figure 1c). The lead compound, MRY D2 (Figure 1a), induced the formation of large ABs at 128 µg/mL (Figure 1c), phenocopying the PEN-induced persistence phenotype (Figure 1c). This observation supports our hypothesis that MRY D2 targets chlamydial MraY and thereby affects the cell division machinery in the cell-wall-lacking minimalist endobacterium.

Compared with MRY D2, all muraymycin derivatives had a lipophilic C_{15} side chain (Figures 1d and S1) added to their peptidic moiety. Each derivative had further modifications in the peptidic moiety, and some were configuration isomers (MRH-22/23; MRH-38/-76; Figures 1d and S1). Except for MRH-92, treatment with all other muraymycin derivatives led to an enlarged chlamydial cell phenotype (Figure S2). However, the phenotype was induced at lower concentrations compared with the lead compound: MRH-22, -38, and -82 had an MIC of 64 µg/mL, while MRH-23, -25, and -76 had an MIC of 32 µg/mL (Figure 1d). Since these derivatives induced the phenotype at lower concentrations, the addition of the lipophilic side chain seemed to enhance the anti-chlamydial activity. Furthermore, the anti-chlamydial activity was increased two-fold for the derivatives MRH-23 and -76, which had an *S*- instead of an *R*-configuration in the bond linking the acylated L-leucine to the peptidic moiety.

The derivative MRH-92 did not induce the formation of aberrant chlamydial cells even at the highest tested concentration of 64 µg/mL (Figure S2). The phenotype of the inclusions resembled that of the dimethyl sulfoxide (DMSO) vehicle control (Figure 1c). We could not explore whether higher concentrations of MRH-92 might have led to the abnormal phenotype observed for the other muraymycins due to host cell cytotoxic effects at concentrations > 64 µg/mL (Table S1).



(d)

Class	Compound	Lipophilic side chain	Modification peptidic moiety compared to MRY D2	Configu-ration	MIC (µg/mL)
Muray-mycins	MRY D2	-	-	*S*	128
	MRH-22	Yes	L-Orn instead of L-*epi*-Cpm	*R*	64
	MRH-23	Yes	L-Orn instead of L-*epi*-Cpm	*S*	32
	MRH-25	Yes	L-Cpm instead of L-*epi*-Cpm	*S*	32
	MRH-38	Yes	Only L-Leu retained	*R*	64
	MRH-76	Yes	Only L-Leu retained	*S*	32
	MRH-82	Yes	Phenyl ring instead of L-Val and L-*epi*-Cpm	*S*	64
	MRH-92	Yes	MRH-23 lacking parts of peptidic moiety	*S*	> 64
Capraza-mycins	cCPZ	Yes	No peptidic moiety	-	16

Figure 1. Effects of muraymycin (MRY) D2, its derivatives, and carbacaprazamycin (cCPZ) on *C. trachomatis*. (**a**) The lead compound MRY D2 consists of a nucleoside and peptidic moiety made up of L-Val, L-*epi*-capreomycidine (Cpm), and L-Leu. The bond between the latter two has an *S*-configuration. (**b**) HEp-2 cells were infected with *C. trachomatis* D/UW-3/CX, the compounds were added at 2 h post infection (hpi), and anti-chlamydial activity was analyzed at 30 hpi by fluorescence

microscopy. (**c**) The vehicle control with dimethyl sulfoxide (DMSO) showed an active infection, 100 U/mL penicillin (PEN) G induced persistence visible as enlarged, aberrant bodies (ABs), while 0.5 µg/mL ciprofloxacin (CIP) was bactericidal. MRY D2 is shown at the minimal inhibitory concentration (MIC; 128 µg/mL), at which the formation of aberrant cells was induced; cCPZ had the lowest MIC of 16 µg/mL. Eukaryotic host cell cytoplasm from representative images was labeled with Evans Blue (red), genomic desoxyribonucleic acid (DNA) with 4′,6-diamidino-2-phenylindole (DAPI; blue), and chlamydial lipopolysaccharide with fluorescein (green). Representative images of three experiments. Scale bar: 10 µm. (**d**) Overview of the structural features of muraymycin derivatives (MRH) and cCPZ compared with MRY D2. MRH-22 and -23 have an L-ornithine (L-Orn). Compared with MRY D2, the MICs of muraymycin and caprazamycin derivatives were lower, ranging between 16 µg/mL and 64 µg/mL. Data obtained from three experiments. MRH-92 did not induce the formation of enlarged chlamydial cells.

In addition, we analyzed the effect of another nucleoside antibiotic on *C. trachomatis*, the caprazamycin-derivative carbacaprazamycin (cCPZ; described in [49]) (Figure S1). Like most of the tested muraymycins, cCPZ induced the formation of enlarged, aberrant chlamydial cells with an MIC of 16 µg/mL, the lowest MIC measured in this study (Figure 1c,d).

Taken together, the majority of muraymycins and cCPZ induced the formation of enlarged *C. trachomatis* cells. In free-living bacteria, these antibiotics inhibit MraY and arrest cell wall biosynthesis [45,47–50]. Based on our cell culture data obtained with *C. trachomatis*, we propose that the natural compounds also block cell division in this cell-wall-lacking non-model bacterium through interference with transferase MraY.

2.2. Muraymycins Induce the Formation of Enlarged Cells in Wolbachia

Chlamydia and *Wolbachia* are both intracellular bacteria with reduced genomes and modified PGN synthesis and cell division machineries. We showed that muraymycins induce the formation of enlarged cells in *C. trachomatis*; thus, we wanted to analyze if there are similar effects on the endosymbiont *Wolbachia* of *Aedes albopictus* (mosquito) cells. First, we assessed the cytotoxicity of the compounds toward the insect cells by quantitative real-time polymerase chain reaction (qPCR) to detect the insect *actin B* gene. Infected *A. albopictus* insect cells were cultured with varying concentrations of the derivatives or with the protein biosynthesis inhibitor DOX as a positive control for nine days. Most of the compounds were detrimental to the host cells; however, MRH-76 and -92 were non-toxic at the tested concentration range.

Therefore, we analyzed the activity of these two muraymycins using fluorescence microscopy and by measuring bacterial depletion by qPCR to detect the *16S rDNA* gene from *Wolbachia*, normalized to the *actin B* gene from *A. albopictus*. As *Wolbachia* grow more slowly than *Chlamydia*, a longer treatment time was needed to see potential effects of the inhibitors [53]. Our microscopy data showed that in untreated and DMSO-treated insect cells, *Wolbachia* were distributed throughout the host cell cytoplasm, seen as small green fluorescent foci (Figure 2a). Whilst treatment with 4 µg/mL DOX led to a depletion of the bacteria from the host cells (Figure 2c), treatment with 8 µg/mL MRH-76 led to the formation of enlarged wolbachial cells (Figure 2a; exemplary cells indicated by white squares), similar to the fosfomycin-induced *Wolbachia* phenotype [33]. Interestingly, enlarged *Wolbachia* also appeared upon treatment with 32 µg/mL MRH-92 (Figure 2a), which had no effect on *C. trachomatis* (Figure 1c).

The diameter of *Wolbachia* cells was determined from the microscopy images, and a significant increase was found for 8 µg/mL MRH-76 as well as for 8 µg/mL and 32 µg/mL MRH-92 compared with the DMSO control (Figure 2b). While control cells had a median diameter of 1.13 µm, cells treated with 8 µg/mL MRH-76 were 37% larger (1.54 µm), and those treated with 8 µg/mL and 32 µg/mL MRH-92 were 13% (1.28 µm) and 23% (1.39 µm) larger, respectively. The proportion of enlarged cells was 24% for the control, 82% for 8 µg/mL MRH-76, and 53% and 65% for 8 µg/mL and 32 µg/mL MRH-92, respectively.

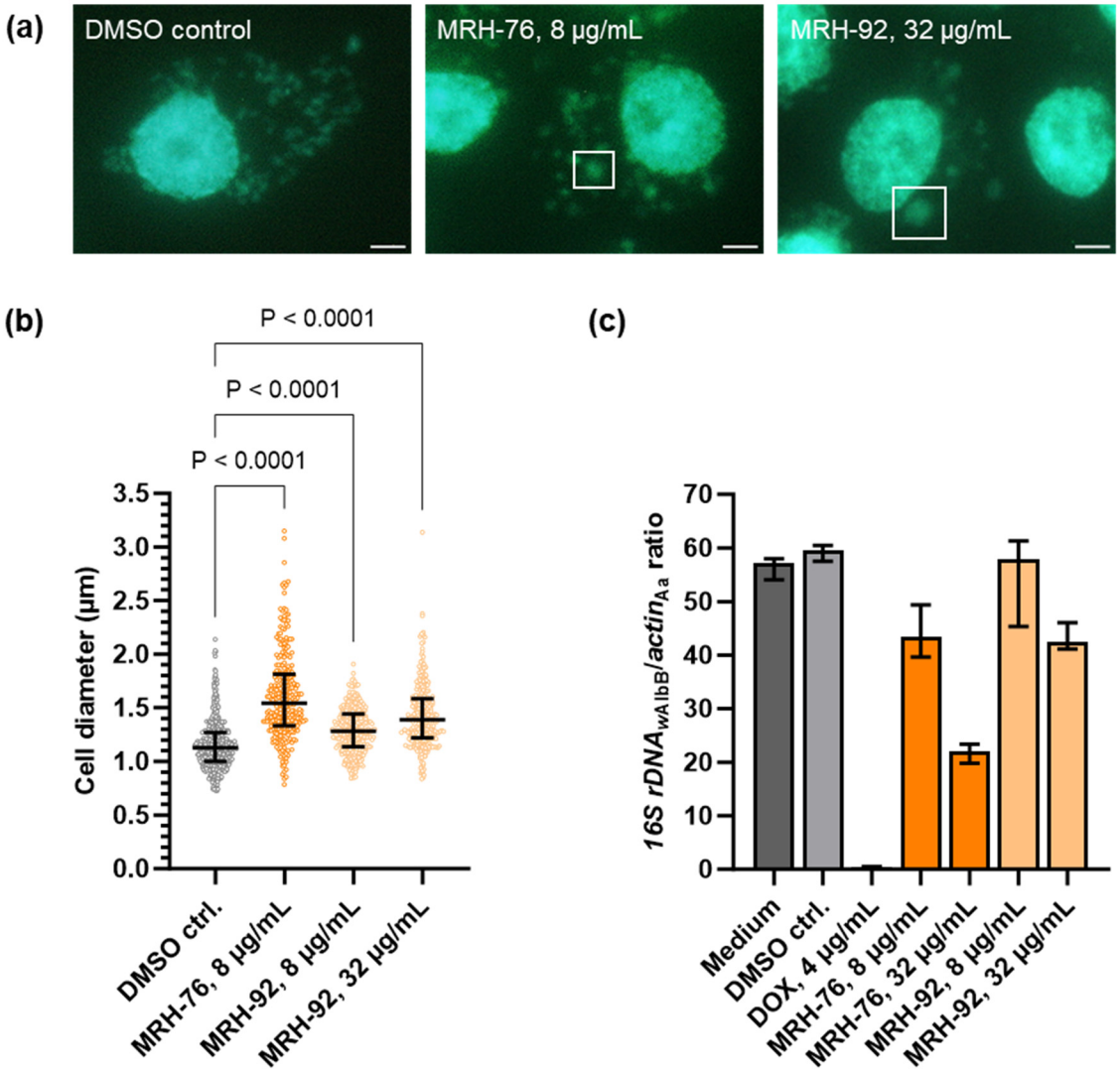


Figure 2. Effects of the muraymycin derivatives MRH-76 and -92 on *Wolbachia* strain B of *Aedes albopictus*. (a) *A. albopictus* C6/36 cells infected with *Wolbachia* strain B of *A. albopictus* were cultured for 9 days with or without the compounds at varying concentrations. Treatment with 8 µg/mL MRH-76 or 32 µg/mL MRH-92 induced the formation of enlarged *Wolbachia* cells (exemplary cells indicated by white squares). Genomic DNA was stained with DAPI (green). Scale bar = 2 µm. (b) Using ZEN 3.5 (blue edition) with the image analysis module, the diameter of untreated and treated *Wolbachia* from 3 to 4 images was determined, shown as median ± interquartile ranges. For statistical analysis, a Kruskal–Wallis test was applied; $p < 0.0001$. $n = 663$ (control), 294 (MRH-76, 8 µg/mL), 348 (MRH-92, 8 µg/mL), and 298 (MRH-92, 32 µg/mL) cells. The cell diameter of *Wolbachia* cells treated with 8 µg/mL MRH-76, as well as of those treated with 8 µg/mL and 32 µg/mL MRH-92, was significantly larger compared with the vehicle control. (c) The depletion of *Wolbachia* from infected and treated C6/36 cells was measured after 9 days by extracting genomic DNA and measuring the ratio of *Wolbachia 16S rDNA* and *A. albopictus actin B* by qPCR; the median ± interquartile range from three experiments is shown. *Wolbachia* were nearly completely depleted by 4 µg/mL doxycycline (DOX), the positive control, whereas treatment with MRH-76 and MRH-92 led to a concentration-dependent reduction of *Wolbachia*, with 32 µg/mL MRH-76 showing a >50% reduction compared with the negative DMSO control.

The relative bacterial quantity was measured by qPCR and revealed that *Wolbachia* were reduced in a concentration-dependent manner compared with the DMSO control. (Figure 2c). While treatment with 8 µg/mL MRH-92 did not affect the abundance of the

bacteria, 32 µg/mL led to a 24% reduction. The same decrease was seen for cells treated with 8 µg/mL MRH-76, whereas 32 µg/mL MRH-76 reduced the intracellular organisms by 68% (Figure 2c). Compared with doxycycline, the muraymycin derivatives were less potent, requiring concentrations higher than 4 µg/mL to achieve depletion of *Wolbachia* over the same treatment time (Figure 2c).

As the treatment of *Wolbachia* with muraymycin derivatives had similar effects to fosfomycin, which targets the first enzyme of UDP-Mur*N*Ac-pentapeptide synthesis, our results support MraY as the target of muraymycins in *Wolbachia*.

2.3. Muraymycin D2 and the Derivative MRH-92 Inhibit the Chlamydial Transferase MraY In Vitro

As shown above, muraymycins induced the formation of enlarged cells in both intracellular species *C. trachomatis* and *Wolbachia*. Therefore, we hypothesized that MRY D2 and the tested derivatives target the transferase MraY (Figure 3a) in *Wolbachia* and *Chlamydia* as in free-living bacteria [45,47–50,54]. We previously showed that recombinant purified MraY of *C. pneumoniae* and *Wolbachia* endosymbiont from *B. malayi* exhibit activity in vitro, leading to the formation of lipid I using bactoprenol and UDP-Mur*N*Ac-pentapeptide [32]. Inhibition of MraY by compounds such as MRY D2 would lead to a depletion of lipid I and thereby block the synthesis of new PGN material. As a result, cell division would be arrested, similar to that seen following treatment with other antibiotics such as PEN G and fosfomycin in *Chlamydia* and *Wolbachia*, respectively [14,33]. To further support the hypothesis that inhibition of MraY by MRY D2 and its derivatives caused the formation of enlarged cells, we performed MraY enzyme inhibition assays.

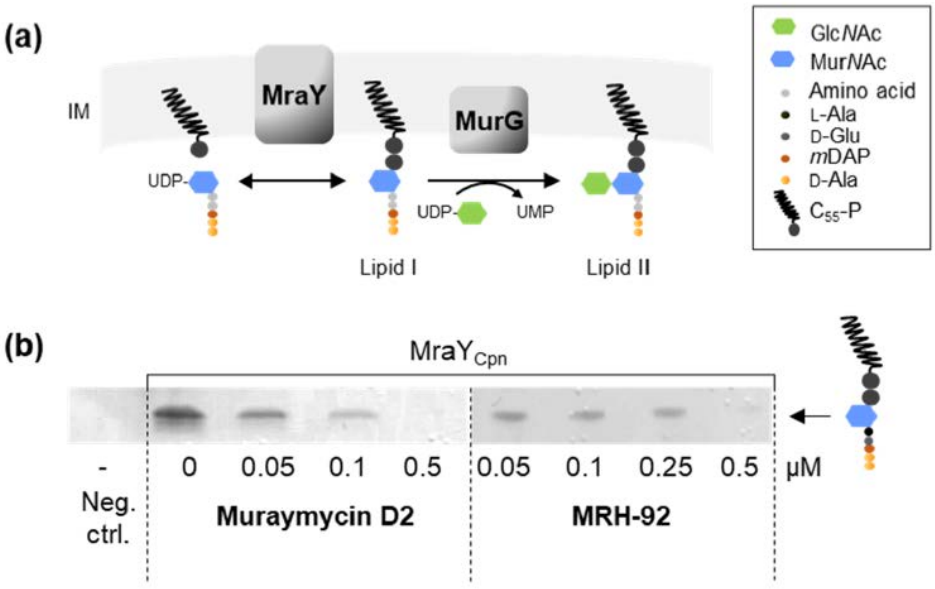


Figure 3. Formation of lipid I by the phospho-Mur*N*Ac-pentapeptide transferase MraY is inhibited by the muraymycins MRY D2 and MRH-92. (**a**) *C. pneumoniae* and *Wolbachia* spp. MraY use the precursor molecules undecaprenyl-phosphate (C_{55}-P) and uridine diphosphate (UDP)-*N*-acetylmuramoyl(Mur*N*Ac)-pentapeptide to form lipid I, which is glycosylated with *N*-acetylglucosamine (Glc*N*Ac) by MurG, yielding lipid II. IM: inner membrane; UMP: uridine monophosphate; mDAP: *meso*-diaminopimelic acid; C_{55}-P: undecaprenyl-phosphate. (**b**) Recombinant $MraY_{Cpn}$ was incubated with C_{55}-P, UDP-Mur*N*Ac-pentapeptide, and different concentrations of either MRY D2 or MRH-92 for 90 min at 37 °C. Reaction products were extracted, separated via thin-layer chromatography, and stained. Formation of lipid I by $MraY_{Cpn}$ was inhibited by both tested inhibitors. MRY D2 showed a concentration-dependent inhibition, with $MraY_{Cpn}$ being completely inhibited at 0.5 µM. MRH-92 also inhibited the enzymatic reaction at 0.5 µM.

C. pneumoniae MraY was heterologously overexpressed in *E. coli*, purified via His-tag affinity chromatography, and inhibition of the enzymatic activity was analyzed by visualizing the reaction products via thin-layer chromatography. We conducted $MraY_{Cpn}$ inhibition assays by incubation with either MRY D2 or MRH-92 at concentrations between 0.05 µM and 0.5 µM, as the compounds did not need to traverse four membranes as in the cell culture-based experiments, and lower concentrations could be used. Both compounds completely abolished enzymatic activity at 0.5 µM (Figure 3b), indicating that $MraY_{Cpn}$ is the target of MRY D2 and MRH-92.

2.4. In Silico Analysis Reveals That Chlamydia and Wolbachia MraY Retain All Amino Acid Residues Important for Binding of UDP-MurNAc-Pentapeptide and Muraymycins

To further support the hypothesis that MraY of *Chlamydia* and *Wolbachia* is inhibited by muraymycins, we performed in silico studies. These included protein sequence alignments and 3D-protein model predictions with phyre2 created for MraY of *C. trachomatis* (Ctr), *C. pneumoniae* (Cpn), and *Wolbachia* endosymbionts of *A. albopictus* (*w*AlbB) and *B. malayi* (*w*Bm) against crystal structures of *A. aeolicus* (Aae) MraY. The $MraY_{Aae}$ active site has been well studied [55–58] in the apo form where the cofactor Mn^{2+} was exchanged for Mg^{2+} [58], as well as in complex with MRY D2 [50]. Additionally, crystallization with cCPZ led to further characterization of residues contributing to the binding of the lipophilic side chain [54].

Our analysis revealed that the MraY orthologs of *Chlamydia* and *Wolbachia* have varying similarities compared to $MraY_{Aae}$. MraY of *w*AlbB and *w*Bm have the highest protein sequence identities with 41% and 40%, respectively, followed by $MraY_{Cpn}$ with 37% and $MraY_{Ctr}$ with 33%, respectively (Table S2). As for $MraY_{Aae}$ [58], our protein models predicted ten transmembrane domains (TM) for the transmembrane enzymes $MraY_{Ctr/Cpn/wAlbB/wBm}$ (Figures 4 and 5). The active sites are predicted to be located at the outside of the proteins, facing the cytoplasm, and are comprised of TM3, TM8, and the cytoplasmic loop E (Figures 4 and 5), as shown for apo$MraY_{Aae}$ [58]. In $MraY_{Aae}$, the four essential catalytic residues are D117, D118, D265, and H324 (Figures 4 and 5; indicated in orange) [56–58]. Several analyses with $MraY_{Aae}$ indicated that D117 is involved in binding the phosphate moiety of the substrate C_{55}-P [57,58], and D265 was shown to be involved in coordinating Mg^{2+} in apo$MraY_{Aae}$ [58]. Our alignment of $MraY_{Ctr/Cpn/wAlbB/wBm}$ with $MraY_{Aae}$ revealed that all four essential catalytic residues are highly conserved in *Chlamydia* and *Wolbachia*: residues D100, D101, D243, and H301 in $MraY_{Ctr}$; D110, D111, D256, and H314 in $MraY_{Cpn}$; and D94, D95, D229, and H293 in $MraY_{wAlbB/wBm}$ (Figure 5).

Crystal structures of $MraY_{Aae}$ bound to MRY D2 showed that MRY D2 did not interact with the three catalytic aspartic acid residues of the active site but, rather, formed a water-mediated hydrogen-bonding network with H324 and H325, occupying the binding site for UDP-Mur*N*Ac-pentapeptide [50]. Upon MRY D2 binding, a conformational change of $MraY_{Aae}$ took place, where the active site was reshaped forming a nucleoside-binding and a peptide-binding pocket, which still faced the cytoplasm (Figure 4) [50,58]. The lipophilic side chain of cCPZ was bound in a hydrophilic cleft (Figure 4) [54,58]. Thus, the mode of inhibition of $MraY_{Aae}$ by MRY D2 and acylated derivatives was competitive for the natural substrate UDP-Mur*N*Ac-pentapeptide regarding the nucleotide-binding site but non-competitive for C_{55}-P [47,50,54].

As in $MraY_{Aae}$, our predictions for $MraY_{Ctr/Cpn/wAlbB/wBm}$ identified that the residues contributing to the binding of MRY D2 and the lipophilic side chain are located on the outside of the proteins and face the cytoplasm and hydrophobic cleft, respectively (Figure 4). Furthermore, $MraY_{Ctr/Cpn/wAlbB/wBm}$ seem to change their conformation as the active sites are wider in our respective predictions, leading to the formation of uracil and peptidic side-chain binding pockets by moving the residues for binding closer together (Figure 4), as shown for $MraY_{Aae}$ [50].

Antibiotics **2024**, *13*, 421

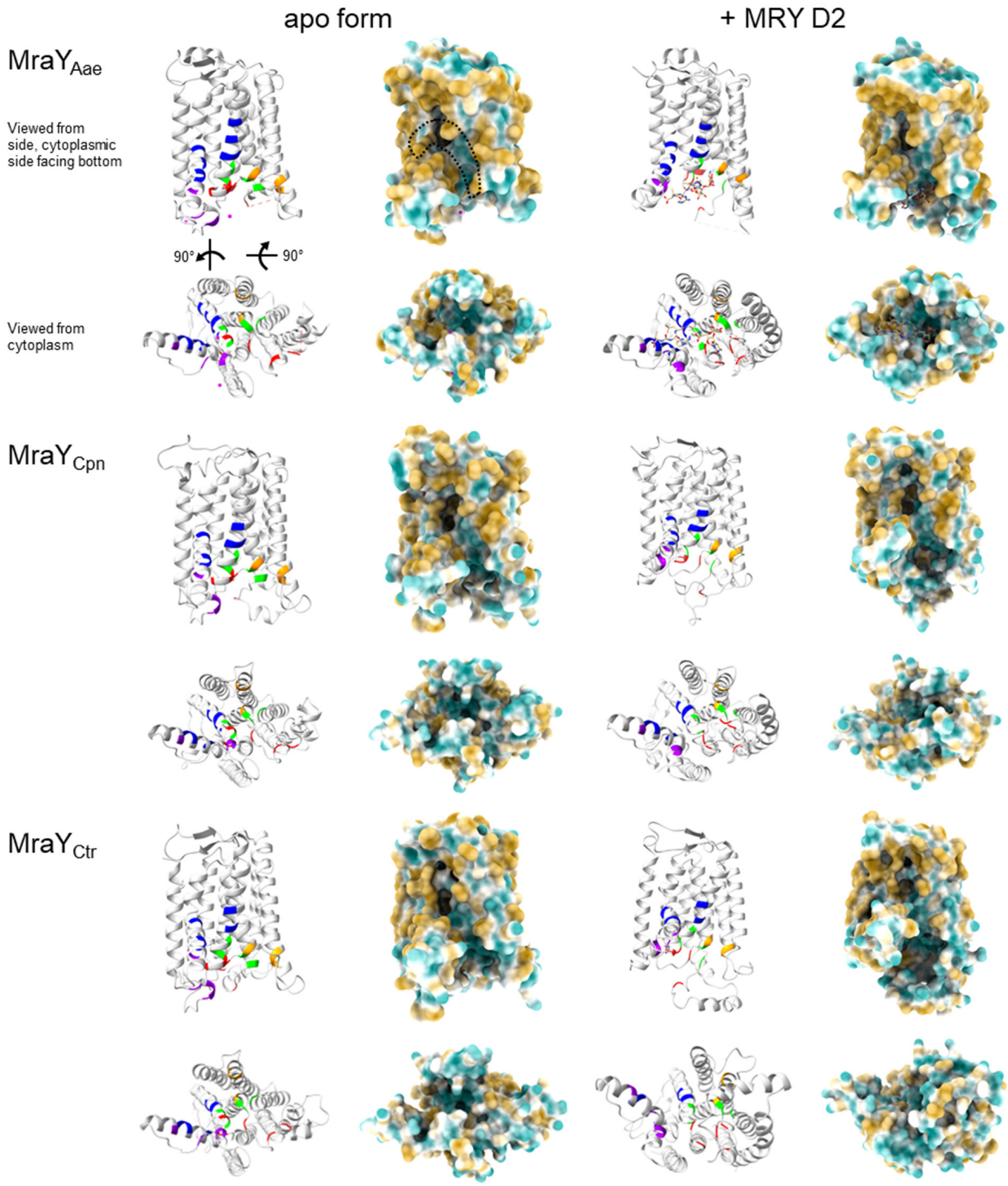


Figure 4. *Cont.*

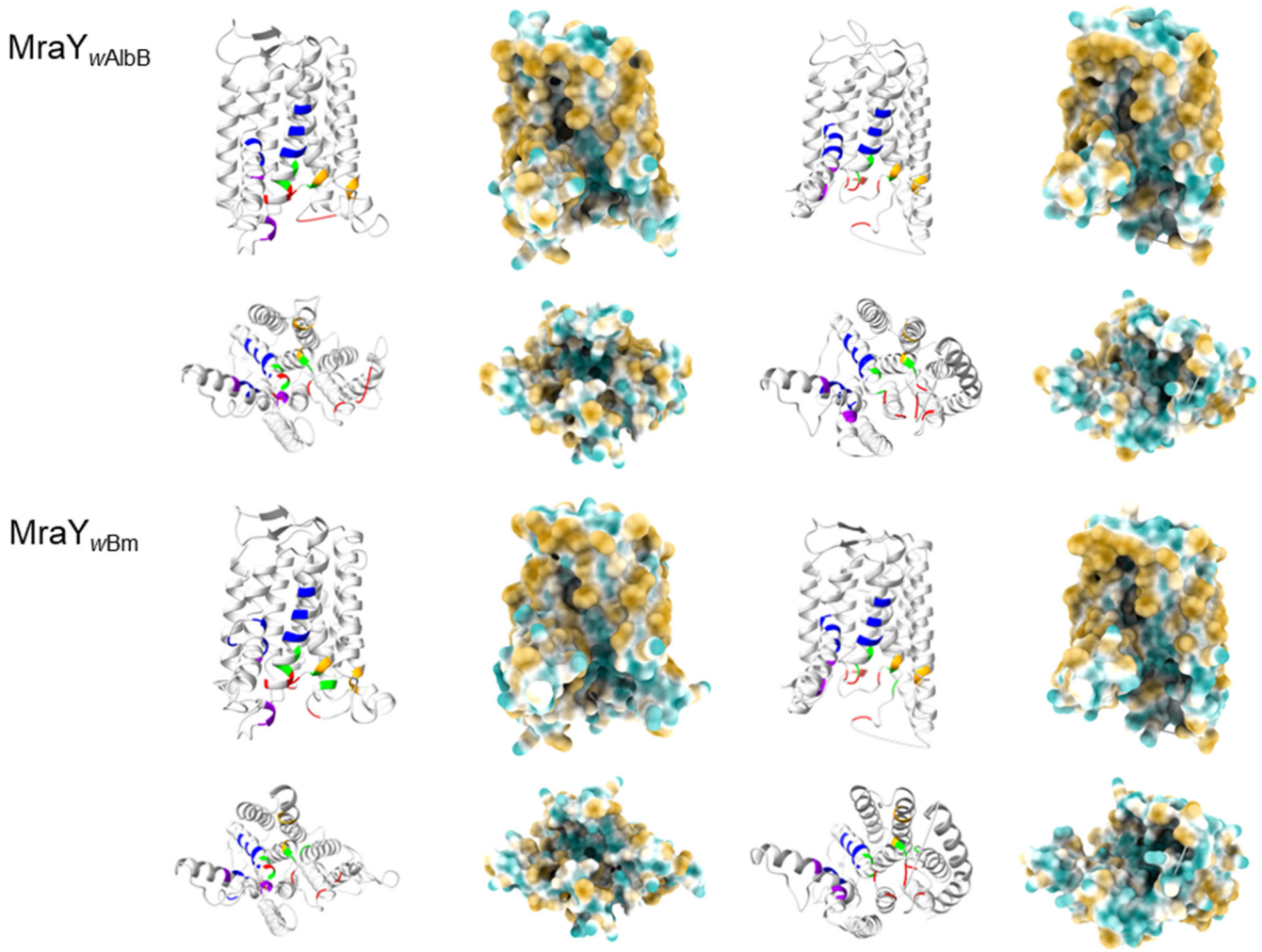


Figure 4. Structural comparison of *A. aeolicus* apoMraY and MraY bound to MRY D2, and predictions of *C. pneumoniae*, *C. trachomatis*, and *Wolbachia* endosymbionts of *A. albopictus* and *B. malayi* MraY and MraY bound to MRY D2. Bacterial strains and primary protein accession numbers: *A. aeolicus* VF5 (Aae, O66465), *C. trachomatis* D/UW-3/CX (Ctr, O84762), *C. pneumoniae* GiD (Cpn, A0A0F7WR30) and *Wolbachia* endosymbiont of *A. albopictus* (*w*AlbB, A0A4S2QUK2) and *B. malayi* (*w*Bm, Q5GRZ3). MraY structure predictions were made by pyhre2 against apoMrY$_{Aae}$ chain A [58] or MraY$_{Aae}$ crystallized with MRY D2 [50] with 100% confidence. Proteins are viewed from the side (top: periplasm; bottom: cytoplasm) or turned by 90° + 90° and viewed from the cytoplasm. Amino acid residues of the active site (orange), Mn^{2+} ions (representative for Mg^{2+}; grey), Ni^{2+} ions (magenta), as well as residues contributing to binding of uracil (red), 5′-aminoribose (green), and the peptidic side chain (violet) from MRY D2 and the acyl moiety of cCPZ (blue), are highlighted as in [50,58]. Surface hydrophobicity is indicated from hydrophobic (brown) to hydrophilic (turquoise). The hydrophobic groove (dashed line) of apoMraY$_{Aae}$ is visualized as in [58]. The phyre2 predictions revealed that MraY$_{Ctr/Cpn/wAlbB/wBm}$ have similar secondary and tertiary structures compared to apoMraY$_{Aae}$. The active sites face the cytoplasm. In MraY$_{Ctr/Cpn/wAlbB/wBm}$, the uracil, 5′-aminoribose, and peptidic side chains face the cytoplasm, residues contributing to the binding of the cCPZ acyl chain are located at the surface of the protein groove as in apoMraY$_{Aae}$. Residues V266 and I267 of MraY$_{wAlbB}$, contributing to acyl moiety binding in MraY$_{Aae}$, could not be predicted.

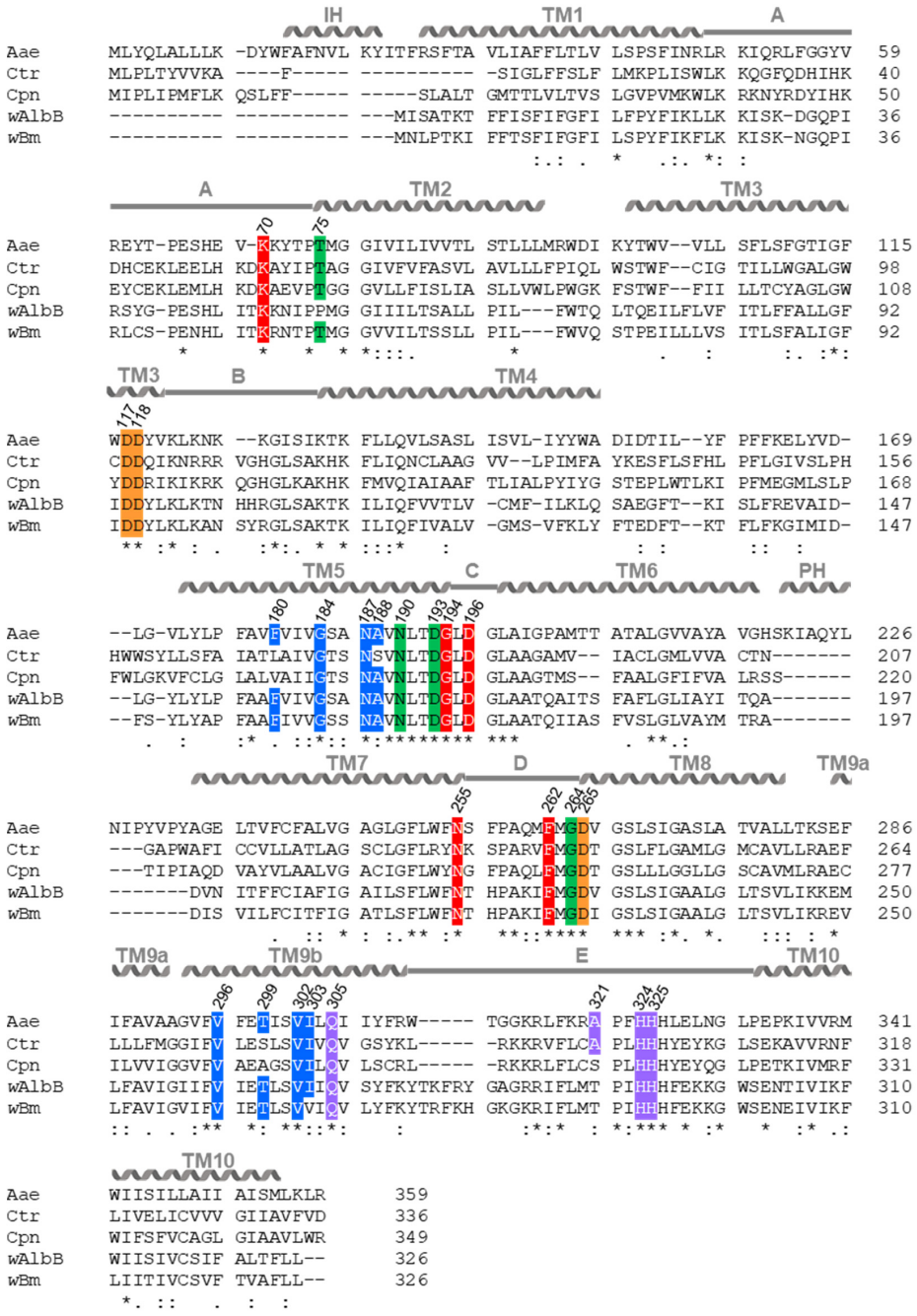


Figure 5. Sequence alignment of MraY from *A. aeolicus*, *C. trachomatis*, *C. pneumoniae*, and *Wolbachia*. Bacterial strains and primary protein accession numbers: *A. aeolicus* VF5 (Aae, O66465), *C. trachomatis* D/UW-3/CX (Ctr, O84762), *C. pneumoniae* GiD (Cpn, A0A0F7WR30) and *Wolbachia* endosymbiont of *A. albopictus* (*w*AlbB, A0A4S2QUK2) and *B. malayi* (*w*Bm, Q5GRZ3). Alignments were performed with Clustal Omega, the position of specific residues is indicated for $MraY_{Aae}$. Compared with $MraY_{Aae}$ [56–58], the three proposed catalytical aspartic acid residues (orange) and histidine (purple) residues within the active site of $MraY_{Ctr/Cpn/wAlbB/wBm}$ are highly conserved. $MraY_{Aae}$ residues contributing to the binding of MRY D2 are indicated based on the respective MRY D2 moieties, which are uracil (red), 5′-aminoribose (green), and the peptidic side chain (violet) [50,54]. The acyl moiety of cCPZ is bound in a hydrophobic groove in $MraY_{Aae}$ (blue) [54]. With a few exceptions, these residues are also conserved in $MraY_{Ctr/Cpn/wAlbB/wBm}$. $MraY_{Aae}$ contains an interfacial helix (IH), ten transmembrane domains (TM), one periplasmic helix (PH), and five cytoplasmatic loops (A–E). Conservation grade: fully conserved (*), strongly similar properties (:), weakly similar properties (.).

Our models for $MraY_{Ctr/Cpn/wAlbB/wBm}$ further showed that the majority of residues contributing to the binding of uracil, 5′-aminoribose, and the peptidic moiety of MRY D2, as well as the binding of a lipophilic side chain, are also highly conserved and are located at the surface of the protein (Figures 4 and 5; indicated in red, green, violet, and blue, respectively). In $MraY_{Ctr}$, the amino acids F180, A188, and T299 of the lipophilic binding site of $MraY_{Aae}$ are not conserved; in $MraY_{Cpn}$, the residues F180 and T299 as well as A321 of the peptidic binding site of $MraY_{Aae}$ are not present. The latter is also lacking in $MraY_{wAlbB/wBm}$. Additionally, in $MraY_{wAlbB}$, T75 of the 5′-aminoribose binding site and in $MraY_{wBm}$, I303 of the lipophilic binding site of $MraY_{Aae}$ are not conserved (Figures 4 and 5).

Together, the high level of conservation at the primary, secondary, and tertiary structural levels gives further evidence for similar enzymatic and inhibitory mechanisms of muraymycins versus $MraY_{Ctr/Cpn/wAlbB/wBm}$ compared with $MraY_{Aae}$. It is likely that MRY D2 and its derivatives fit into the binding pockets of the enzymes and thereby compete with the substrate UDP-Mur*N*Ac-pentapeptide, leading to inhibition of the enzyme and, thus, depletion of lipid I within the cell.

2.5. Muraymycin D2 Eliminates Persistent C. trachomatis Cells from an Established PEN-Induced Persistent Infection upon Addition at the Mid-Stage of the Chlamydial Developmental Cycle

As we have shown, muraymycins target MraY and interfere with the cell division process in both *Chlamydia* and *Wolbachia*, leading to enlarged cells, phenocopying the PEN-induced persistent infection in *Chlamydia* [14] and the fosfomycin-induced phenotype in *Wolbachia* [33]. The mechanism behind the PEN-induced persistence in *Chlamydia* is not entirely understood. Because muraymycins and PEN G have different structural targets in PGN synthesis, we investigated the effects of simultaneous treatment with MRY D2 and PEN G on a chlamydial infection and if MRY D2 has an effect on an established PEN G-induced persistent *C. trachomatis* infection. The latter would also be of interest in the context of the treatment of chlamydial infections in vivo, where enlarged chlamydial cells have been observed and treatment failures with first-choice antibiotics have been reported [6,12,16–18].

To study simultaneous treatment, we added 100 U/mL PEN G and 128 µg/mL of MRY D2 at 2 hpi to *C. trachomatis* D/UW-3/CX-infected cells to analyze any effects of a combined administration (Figure 6a). These experiments revealed that when MRY D2 and PEN G were added simultaneously, inclusions filled with ABs were visible as for PEN G alone (Figure 6b–d).

We next investigated the effects of MRY D2 on an established persistent infection by inducing persistence with PEN G at 2 hpi followed by the addition of MRY D2 at 12 hpi (Figure 6a). At this time point, chlamydial cells are usually in the mid-phase of their developmental cycle and have started replicating [14,51]. In cells treated with PEN G at the beginning of the infection, ABs are visible after 12 h [14]. After the application of MRY D2 at 12 hpi to the PEN G-induced persistent *C. trachomatis* infection, fewer inclusions containing ABs were observed (Figure 6b,d), corresponding to a 70% reduction of the inclusion quantity (Figure 6c). As a control, MRY D2 was also added alone at 12 hpi (Figure 6a) to the active infection at the mid-stage of the chlamydial developmental cycle. Again, 75% fewer inclusions were observed, of which 83% contained EBs/RBs and 8% contained a mixed phenotype (EBs/RBs/ABs) (Figure 6b,c).

In summary, we saw that the mid-stage application of MRY D2 to dividing or persisting *Chlamydia* results in clearance of the inclusions by the host cell.

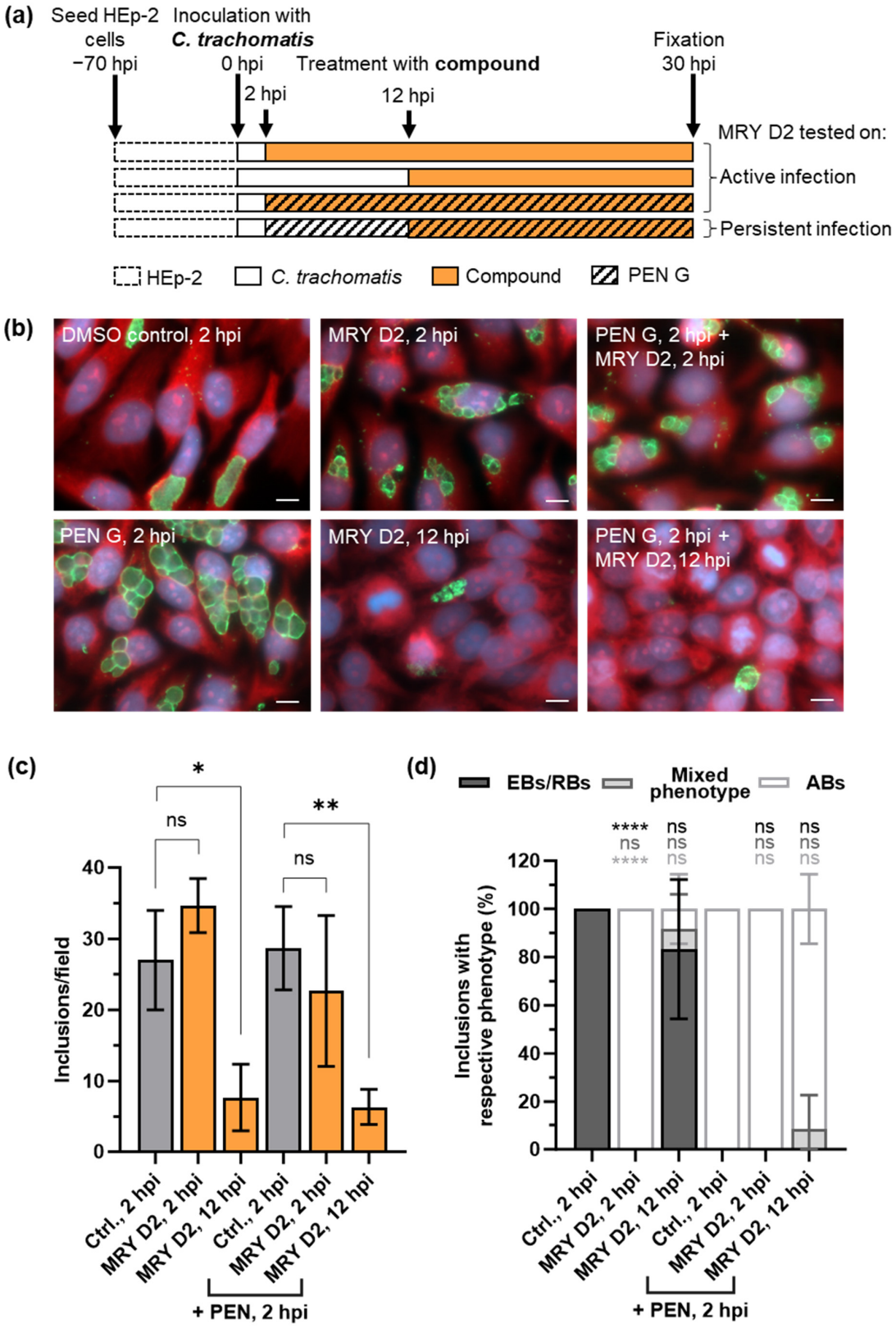


Figure 6. Effect of MRY D2 on PEN G-induced persistent *C. trachomatis* infections. (**a**) HEp-2 cells infected with *C. trachomatis* D/UW-3/CX were treated with 128 µg/mL MRY D2 at 2 hpi or 12 hpi to analyze the effect on active infection. Additionally, 128 µg/mL MRY D2 and 100 U/mL PEN G were added simultaneously at 2 hpi. To assess the effect on a PEN G-induced persistent infection, 100 U/mL PEN G was added at 2 hpi, and 128 µg/mL MRY D2 was added at 12 hpi. Anti-chlamydial

activity was analyzed at 30 hpi by fluorescence microscopy. (**b**) Eukaryotic host cell cytoplasm from representative images was labelled with Evans Blue (red), genomic DNA with DAPI (blue), and chlamydial lipopolysaccharide with fluorescein (green). Scale bar: 10 µm. (**c**) Mean ± SD of inclusions/field and (**d**) the relative number of inclusions filled with either elementary bodies (EBs)/reticulate bodies (RBs), ABs, or a mixed phenotype, analyzed from three images. An unpaired, two-tailed Student's *t*-test was performed against the respective vehicle control. ns: not significant, $p > 0.05$; *: $p = 0.05$ to 0.01; **: $p = 0.01$ to 0.001; ****: $p < 0.0001$. In comparison with the DMSO vehicle control, the addition of PEN G at 2 hpi induced persistence, characterized by aberrant bodies. Early application of MRY D2 at 2 hpi ($p < 0.0001$) and simultaneous application with PEN G at 2 hpi induced the formation of ABs. Application of MRY D2 at mid-phase (12 hpi) did not lead to the formation of ABs, but fewer inclusions were observed ($p = 0.0166$). This was also visible if persistence was induced by PEN G at 2 hpi followed by MRY D2 addition at 12 hpi ($p = 0.0037$).

3. Discussion

The endobacteria *Chlamydia* spp. and *Wolbachia* spp. are of medical importance. Depending on the serovar, *C. trachomatis* can cause eye infections and infections of the ano-urogenital tract. Chronic or untreated infections can have detrimental long-term effects, e.g., blindness (serovar A-C) [1] or infertility in women (serovar D-K) [3,4]. Genital chlamydial infections are treated with DOX or AZI [5]; however, an 8% treatment failure rate is reported [8,9], which might occur due to re-infection from the gastro-intestinal tract [10] or persistent *C. trachomatis* cells [6,12]. Persisting chlamydial cells were shown to be less susceptible to DOX and AZI in murine models and cell culture experiments [11,16–18]. *Wolbachia* reside in the filarial nematodes *W. bancrofti*, *B. malayi*, *B. timori*, and *O. volvulus* [19,20]. These nematodes cause the neglected tropical diseases lymphatic filariasis (lymphedema or hydrocele) and onchocerciasis (severe skin disease or vision loss). Anti-wolbachial treatment with DOX or rifampicin is the only effective and safe treatment against adult worms as *Wolbachia* are obligate endosymbionts that support filarial development, growth, and survival [23,24,27]. Overall, contraindications for these antibiotics, including long treatment regimens for the anti-wolbachial treatment of filariasis, and treatment failure in *Chlamydia* require new strategies to fight these infections.

In this study, we explored the activity of the nucleoside antibiotics muraymycins and caprazamycins against *Chlamydia* and *Wolbachia*. Muraymycins and caprazamycins share a core structure comprised of glycyluridine, to which an aminoribose is attached at the 5′ position [43,44,46]. In MRY D2, an *N*-alkylamide linker is attached to the 6′ position of the nucleoside and connects the core structure with a peptidic moiety consisting of L-Leu, L-*epi*-capreomycidine, and L-Val [43]. Carbacaprazamycins, which have an acyl chain linked directly to the diazepanone ring, are chemically more stable derivatives of caprazamycin [49]. Nucleoside antibiotics display structural similarity to UDP-Mur*N*Ac-pentapeptide, the natural substrate of the transferase MraY, which catalyzes the formation of lipid I. This is the penultimate precursor molecule of bacterial PGN. Due to the structural similarity of nucleoside antibiotics and UDP-Mur*N*Ac-pentapeptide, the antibiotics are competitive for the MraY binding site of the substrate [47,50,54] and thereby inhibit enzymatic activity, as shown for multiple species, e.g., *B. subtilis* and *S. aureus* [45,48,49]. Binding of nucleoside antibiotics to MraY blocks lipid I formation and halts bacterial PGN synthesis.

Inside their intracellular niches, *Chlamydia* and *Wolbachia* are protected against osmotic stress [32], making a canonical cell wall redundant. However, both genera have retained all genes for *de novo* synthesis of the ultimate PGN building block, lipid II [32,33]. We previously hypothesized that lipid II synthesis is essential for cell division of *Chlamydia* and *Wolbachia* and demonstrated functional conservation of the transferase MraY and the transglycosylase MurG, which catalyze the formation of lipid I and lipid II, respectively [32]. Furthermore, some enzymes involved in the assembly, modification, and recycling of PGN are conserved [33,34]. Later, it was shown that *Chlamydia* synthesizes a transient PGN ring that facilitates proper cell division initiated by a specialized budding mechanism [29,30,35,36], whereas the exact PGN structure in *Wolbachia* is still unknown. However, research in this

field proposes a reduced PGN-like structure [31–33]. Based on these observations, we postulated that nucleoside antibiotics, including muraymycins, exhibit antibacterial activity against the minimalist endobacteria *Chlamydia* and *Wolbachia* via inhibition of MraY, leading to interference of the PGN synthesis and cell division machineries.

To investigate the effects of the nucleoside antibiotics against *C. trachomatis*, we tested MRY D2, seven muraymycin derivatives, and cCPZ, a caprazamycin derivative [47–49]. In contrast to MRY D2 (Figure 1a), all derivatives possessed a lipophilic C_{15} side chain (Figures 1d and S1). Furthermore, the L-*epi*-capreomycidine in the peptidic moiety of MRY D2 was exchanged for L-capreomycidine in MRH-25 and for L-ornithine in MRH-22 and -23, respectively. In MRH-38 and -76, the whole peptidic moiety was deleted, while in MRH-82 and MRH-92, the peptidic moiety was reduced to a phenyl group and an L-ornithine, respectively, (Figures 1d and S1). The compounds MRH-23/MRH-25 and MRH-38/MRH-76 are configuration isomers. Of note, all compounds retained the uracil, 5′-aminoribose, and 3′ hydroxyl group of the 5′ aminoribose, which are essential for activity, as shown for caprazamycins and lipsidomycins, another class of nucleoside antibiotics [59–61].

The tested compounds were applied to *C. trachomatis*-infected cells at an early stage of the chlamydial developmental cycle (Figure 1a), before the bacteria started replicating [14,51]. During the early treatment with MRY D2, the majority of muraymycin derivatives and cCPZ had MICs between 16 µg/mL for cCPZ and 128 µg/mL for MRY D2 (Figure 1d). These MICs were relatively high compared with the MICs of the first-choice antibiotics against uncomplicated chlamydial genital infections, DOX and AZI, which have MICs of <1 µg/mL against *C. trachomatis* serovar D in cell culture [62,63]. All derivatives harboring the lipophilic side chain had more potent anti-chlamydial activity compared with MRY D2 as reflected by their lower MICs (Figure 1d). This is probably due to the increased membrane permeability of the derivatives, as the compounds have to pass four membranes to reach their target MraY in *Chlamydia*. The same effect was also described for free-living pathogens [47].

The stereochemistry of the bond linking the peptidic moiety to the nucleoside core structure (Figures 1d and S1) influenced the activity of the compound, as described in *S. aureus*, *Enterococcus faecalis*, and *Enterococcus faecium* [45,47]. If the bond had an *S*-configuration, as in the isomers MRH-23 and -76 (32 µg/mL), the MIC for *Chlamydia* was two-fold lower compared with the *R*-configuration in the isomers MRH-22 and -38 (64 µg/mL, Figure 1d). Another structural feature that influenced the activity in *S. aureus*, *E. faecalis*, and *E. faecium* were the amino acids of the peptidic moiety. The activity was two- to four-fold higher when the peptidic moiety included L-capreomycidine instead of L-ornithine [47]. This seemed to be less relevant in *C. trachomatis*, as reflected by the same MIC for MRH-25 and -23 (64 µg/mL), which had the same structure but contained L-capreomycidine and L-ornithine, respectively (Figures 1d and S1). Furthermore, other studies revealed the importance of L-leucine of the peptidic moiety for the activity of the derivatives [45,47]. Exchanging the accessory peptidic motif, including L-leucine and the *N*-alkylamide linker, for a C_{12} side chain led to a much weaker activity similar to MRY D2 in *S. aureus*, *E. faecalis*, and *E. faecium* [45,47]. In our study, the muraymycin derivatives with the most reduced structure were MRH-38 and -76, which only retained the acylated L-leucine (Figures 1d and S1). They exhibited similar anti-chlamydial activity (64 µg/mL and 32 µg/mL) as the other derivatives (Figure 1d), highlighting the importance of the peptidic moiety for interacting with the target enzyme and thereby increasing the antibacterial activity of these derivatives. Based on our observations, improved anti-chlamydial activity of a muraymycin is obtained by acylation, which increases the membrane permeability, and by having an *S*-configuration of the bond linking L-leucine to the rest of the peptidic moiety. The lowest MIC measured for *C. trachomatis* was for cCPZ (Figure 1d). As proposed by others, the diazepanone ring might contribute to the higher anti-chlamydial activity by positioning the uridine, 5′-aminoribose, and acyl chain to the respective binding sites in MraY [49,54,59].

Most muraymycins tested in this study induced the formation of enlarged chlamydial cells, with one to a few of these ABs per inclusion (Figures 1c and S2). The phenotype is similar to the one seen in PEN G-induced persistent chlamydial infection (Figure 1c) [14]. Compared with MRY D2 inhibiting the formation of lipid I, PEN G acts downstream of MraY in the chlamydial PGN synthesis pathway (Figures 7 and 8). It binds to the transpeptidases PBP2 and PBP3 and the D,D-carboxypeptidase PBP6 [39,64], thereby inhibiting synthesis of cross-linked PGN and blocking cell division [36–38]. Although PEN G and MRY D2 both halt chlamydial cell division, different processes are likely to be involved at a cellular level in the bacteria. PEN G-induced persistent chlamydial cells try to compensate for the growth defect by upregulating the transcription of several genes important for PGN precursor synthesis, including the UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase MurA and ligases MurD and MurE. However, the transcription level of MraY is not affected [65]. Furthermore, it was shown by fluorescent labeling of PGN that the PGN ring dissociates under PEN G treatment due to continued PGN degradation accompanied by increased shedding of PGN material [38].

While a few small foci of nascent and non-cross-linked PGN are present under PEN G treatment at an early stage of the chlamydial developmental cycle [38], it is assumed that PGN synthesis is not initiated during the early application of muraymycins. First, transcription of most genes involved in lipid II synthesis starts between >8 hpi to ≤16 hpi [66], which is the time during which the first cell division takes place [14,51]. This includes most Mur enzymes and MraY. Only transcription of the non-canonical alanine racemase GlyA, which converts L-Ala into D-Ala [67], and MurF, which ligates the alanine dipeptide to UDP-Mur*N*Ac-tripeptide [68], starts between >3 hpi and ≤8 hpi [66]. These data indicate that the enzymes for lipid II synthesis, including MraY, are newly synthesized by *Chlamydia* after infection of a new host cell. It would then be inhibited by muraymycins directly or soon after the synthesis of the enzyme. Second, the levels of lipid II are predicted to be very low in Gram-negative bacteria compared with other lipids, as shown for *E. coli*, due to the rapid translocation of lipid II to the periplasm and its incorporation into PGN [69]. Thus, even if there are some molecules of lipid I and II synthesized prior to the inhibition by muraymycins, only a few rounds of cell division might be possible. This would lead to inclusions filled with a few chlamydial cells, as seen for most of the tested muraymycins (Figures 1c and S2) and as shown for PEN G (Figure 1c) [14], compared with untreated inclusions filled with ~ 500 chlamydial cells [14,70]. However, the presence of a few ABs per inclusion could also arise from multiple EBs infecting one host cell. Overall, we propose that upon early inhibition of chlamydial MraY by muraymycins, the production of lipid I and thereby lipid II is abolished and PGN synthesis is not initiated, leading to a block in cell division and subsequent enlargement of chlamydial cells lacking PGN (Figure 7a).

The phenotype induced by PEN G in chlamydial cell culture models is commonly referred to as a persistent state, which is characterized by an inhibition of cell division, enlarged chlamydial cells, an inhibited production of infectious progeny, and the reversibility of this phenotype. Two of these four criteria are fulfilled for muraymycins as the chlamydial cells stop dividing and appear aberrant. Whether or not the muraymycins induce a true persistent state in *Chlamydia*-infected cell culture models, which can be reversed after removal of the antibiotic, resulting in the production of new infectious progeny, needs to be addressed in the future.

As muraymycins had an effect on cell division in intracellular *Chlamydia*, we also investigated the effect against the endosymbiont *Wolbachia*. Although no canonical cell wall has been detected in this genus, a PGN-like structure was visualized using the clickable D-alanine analog ethynyl-D-alanine [31]. We have shown that the endobacteria have retained the ability to form lipid II by MraY and MurG [32], and several other genes involved in PGN synthesis are also conserved [33]. Of these, transcription could be proven for many genes from the *Wolbachia* endosymbiont of *A. albopictus*, including RodA, a protein of the shape, elongation, division, and sporulation (SEDS) family, the transpeptidase PBP2, and the carboxypeptidase PBP6a [33]. In general, *Wolbachia* strains have retained at least one

of the SEDS proteins, RodA or FtsW, and at least one monofunctional class B PBP [31,34]. In *Wolbachia* of *B. malayi* and *A. albopictus*, PBP2 and RodA are conserved. Recently, both RodA and FtsW of several Gram-positive bacteria were shown to exhibit monofunctional glycosyltransferase activity [71,72], which would close the gap of the missing glycosyltransferases in *Wolbachia*. Thereby, a minimum of one putative glycosyltransferase and one transpeptidase would be present, which could catalyze the formation of a PGN-like structure in *Wolbachia* [31,34]. Research on cell-wall-targeting antibiotics in *Wolbachia* is helping to elucidate the composition of the PGN synthesis machinery and PGN structure of *Wolbachia* spp.

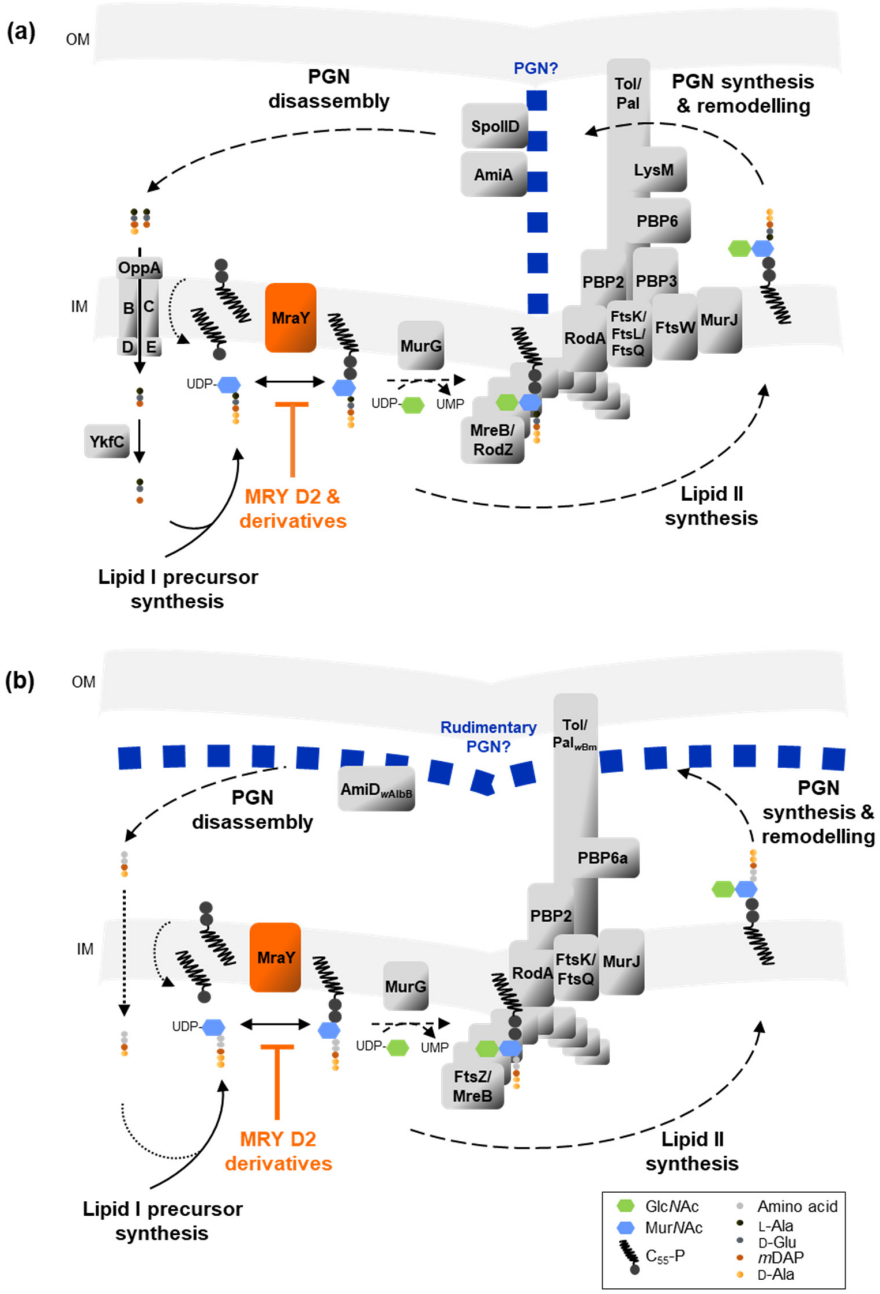


Figure 7. Muraymycins inhibit lipid I formation by binding to MraY and blocking lipid II and peptidoglycan (PGN) synthesis in *C. trachomatis* (a) and in *Wolbachia* of *A. albopictus* and *B. malayi* (b). Enzymes that are solely conserved in *Wolbachia* of *A. albopictus* (*w*AlbB) or *B. malayi* (*w*Bm) are indicated. Dashed lines indicate inhibited/downregulated processes; dotted lines indicate uncharacterized processes. OM: outer membrane; IM: inner membrane.

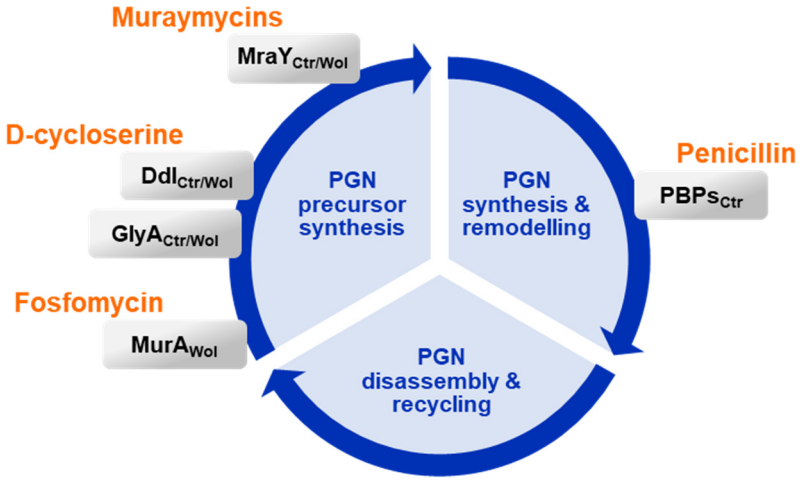


Figure 8. Antibiotic inhibition of enzymes catalyzing important steps in the different PGN synthesis and degradation pathways interrupt the PGN cycle in *C. trachomatis* (Ctr) and *Wolbachia* (Wol), leading to an arrest in cell division.

In this study, the muraymycin derivatives MRH-76 and MRH-92 were tested for their antibacterial activity against *Wolbachia* in cell culture. Both had the same effect: they induced the formation of enlarged *Wolbachia* cells (Figure 2a, b) similarly to muraymycin treatment in *Chlamydia* and resembling the PEN-induced persistent phenotype. Unlike in *Chlamydia*, PEN G does not have an effect on *Wolbachia* [40]; however, the antibiotic fosfomycin, targeting MurA of the lipid II synthesis pathway [73] (Figure 8), also induced the formation of enlarged *Wolbachia* cells [33]. The enlarged cells caused by muraymycins and fosfomycin indicate a block of cell division in *Wolbachia* due to the inhibition of lipid II synthesis [33]. Although the exact PGN structure of *Wolbachia* remains to be described, we propose that PGN synthesis is abolished upon muraymycin treatment (Figure 7b).

Due to the shared similarities between *Chlamydia* and *Wolbachia*, it is conceivable that *Wolbachia* also try to compensate for the growth defect by increasing the transcription of genes involved in PGN precursor synthesis. Furthermore, fewer *Wolbachia* were detected in the host cells compared with untreated infected host cells (Figure 2c). The effect was more pronounced for MRH-76 compared with MRH-92 in regards to the wolbachial median cell diameter and the proportion of enlarged cells. Atwal et al. (2021) observed a similar bacterial depletion compared to the control in the D-cycloserine treatment against *Wolbachia* in *Drosophila melanogaster* [31]. This D-amino acid antibiotic is commonly known to target PGN precursor synthesis by inhibiting alanine racemases and D-alanyl-D-alanine ligases involved in lipid II synthesis (Figure 8). *Wolbachia* has retained and expresses the enzyme Ddl and the alternative D-alanine racemases GlyA and MetC [33], the first two of which are potential targets for D-cycloserine, as shown for *Chlamydia* [67,74]. Whether the depletion of *Wolbachia* by antibiotics targeting lipid II synthesis is an indirect effect of inhibited cell division and less growth over time and/or an active depletion of the bacteria needs to be addressed in the future. However, a similar effect could be expected for muraymycins in vivo in murine models or in patients if the compounds can be absorbed by the tissue, as observed for other *Wolbachia*-depleting antibiotics such as DOX and rifampicin [23,24]. Analyses regarding the reversibility of the phenotype could elucidate whether fosfomycin and muraymycins can induce a persistent-like state in *Wolbachia* as defined for PEN-treated *Chlamydia*.

Since we demonstrated antibacterial activity of muraymycins against *Chlamydia* and *Wolbachia* in cell culture experiments, we next investigated whether this activity is mediated by MraY inhibition. Using inhibition assays performed with recombinant purified $MraY_{Cpn}$, we confirmed the enzymatic inhibition by MRY D2 and MRH-92 (Figure 3b). The enzyme was inhibited at a relatively low concentration compared with the MICs determined for *C. trachomatis*. This difference was also observed for MRY D2, in contrast to its acylated

derivative, which had a 32-fold lower MIC for several *S. aureus* strains [45,47]. The lipophilic acyl chain of the derivative increased the overall lipophilicity of the compound and thus the membrane permeability. The MIC of MRH-92 was > 64 µg/mL (Figure 1d). However, both compounds have to traverse four membranes in *C. trachomatis*-infected host cells to reach its target MraY in *Chlamydia*, a process which could contribute to higher MICs in general. Furthermore, the inhibition of $MraY_{Cpn}$ was not altered by the addition of a palmitoyl chain and further derivatization of the peptidic moiety in MRH-92 (Figures 1d and S1), similar to results obtained in inhibition assays with *S. aureus* MraY [48].

For a deeper insight into the enzymatic function and mode of inhibition, we performed a detailed analysis, which included alignments and protein prediction modeling of MraY from *Chlamydia* and *Wolbachia* in comparison to the well-characterized enzyme of *A. aeolicus* [50,54,58]. We included four strains in the analysis: *C. trachomatis*, *C. pneumoniae*, and *Wolbachia* endosymbionts of the human pathogenic filarial nematode *B. malayi* and of the mosquito *A. albopictus*; the latter was used for the cell culture experiments as *Wolbachia* of *B. malayi* is not culturable.

Our analyses showed that $MraY_{Ctr/Cpn/wAlbB/wBm}$ were highly conserved with $MraY_{Aae}$ (Table S2). Protein predictions against apo$MraY_{Aae}$ [58] revealed that the transmembrane protein $MraY_{Ctr/Cpn/wAlbB/wBm}$ is predicted to have ten transmembrane domains, of which TM3, TM8, and the cytoplasmic loop E form the active site facing the cytoplasm (Figures 4 and 5). In $MraY_{Aae}$, four essential catalytic residues were identified [56–58]. While D265 is involved in coordinating the catalytic Mg^{2+} [58], D117 is proposed to bind to the phosphate of the substrate C_{55}-P [57,58]. All four catalytic residues of $MraY_{Aae}$ are highly conserved in $MraY_{Ctr/Cpn/wAlbB/wBm}$ and are located in the active site (Figures 4 and 5). Due to this, we propose that the endobacterial $MraY_{Ctr/Cpn/wAlbB/wBm}$ function like $MraY_{Aae}$, with the respective aspartic acid residues D100/D110/D94/D94 interacting with the substrate C_{55}-P and the D243/D256/D229/D229 residues coordinating the Mg^{2+}.

Due to the structural similarities of the MraY natural substrate UDP-Mur*N*Ac-pentapeptide and nucleoside antibiotics, the antibiotics are able to bind to and inhibit MraY. One of the best-characterized nucleoside antibiotics is MRY D2, which is competitive for the nucleotide of UDP-Mur*N*Ac-pentapeptide [47,50] and non-competitive for the substrate C_{55}-P [47]. Upon binding of MRY D2 to MraY, a conformational change of the enzyme widens the active site, creating a nucleoside- and peptide-binding pocket. The acyl chain of carbacaprazamycin binds in a hydrophobic cleft of $MraY_{Aae}$. Our sequence comparison and 3D modeling of $MraY_{Ctr/Cpn/wAlbB/wBm}$ against $MraY_{Aae}$ bound to MRY D2 [50] revealed that the amino acid residues important for binding to the uracil, 5′-aminoribose, and peptidic moiety of MRY D2 and the acyl chain of cCPZ are, with a few exceptions, also highly conserved among these four species and face the cytoplasm or the hydrophobic cleft (Figures 4 and 5). In total, eight amino acids contribute to the binding of the lipophilic side chain in $MraY_{Aae}$. In $MraY_{Ctr/Cpn/wAlbB}$, five to seven of these residues are present (Figures 4 and 5). The conserved residues in $MraY_{Cpn}$ are sufficient for the binding of MRH-92 as evidenced by the observed inhibition (Figure 3b). The MRY D2 derivatives are also able to exert antibacterial activity against *C. trachomatis* and *Wolbachia* (Figures 1d and 2). A residue that is not conserved in $MraY_{Cpn/wAlbB}$ is A321 of $MraY_{Aae}$, which facilitates binding to the peptidic moiety of MRY D2. This part of the peptidic moiety is not present in the derivatives MRH-38, -76, and -92 (Figure S1); thus, this missing residue in $MraY_{Cpn}$ and $MraY_{wAlbB}$ is not important for binding these three derivatives. Since MRH-92 and MRY D2 abolished the enzymatic activity of $MraY_{Cpn}$ at the same concentration (Figure 3b), the amino acid exchange from A321 to a serine in $MraY_{Cpn}$ is not detrimental for the inhibitory effect of MRH-92 compared to MRY D2. This also fits with the observation that MRY D2 binding to $MraY_{Aae}$ was most disturbed by D193N and F262A mutations [50], of which both residues are conserved in $MraY_{Ctr/Cpn/wAlbB/wBm}$.

Considering the in vitro inhibition of $MraY_{Cpn}$ by muraymycins and the high level of conservation of residues contributing to binding of the antibiotics in $MraY_{Ctr/Cpn/wAlbB/wBm}$, we postulate a similar mode of inhibition for muraymycins toward chlamydial and wolbachial MraY as for $MraY_{Aae}$: muraymycins harboring the essential uracil moiety, the 5′-aminoribose, and the 3′-hydroxyl group of the 5′-aminoribose [59–61] bind to MraY of *Chlamydia* and *Wolbachia* via the conserved amino acid residues, leading to a conformation change in the enzyme. This binding is competitive for the nucleotide from the natural substrate UDP-Mur*N*Ac-pentapeptide and non-competitive for C_{55}-P.

Based on our observations that muraymycins inhibit chlamydial MraY and thereby block further lipid II formation, we were curious about the effects of MRY D2 on a PEN G-induced persistent infection with *C. trachomatis*. Compared with MRY D2, PEN G acts downstream in the PGN cycle by inhibiting PGN transpeptidation and remodeling (Figure 8). A persistent chlamydial state in cell culture models can be induced by β-lactams or stress factors such as nutrient starvation or interferon-γ [13,14]. However, the enlarged chlamydial cell phenotypes can also be detected in vivo in tissue obtained from patients [6,12]. Several studies conducted in vitro showed evidence for β-lactam-induced ABs being less susceptible to treatment with the first-choice antibiotics AZI and DOX [16–18]. Reduced susceptibility of persisting chlamydial cells could be one important factor in treatment failures in infected patients, resulting in reoccurring infections with potential long-term effects. Therefore, it is of importance to understand how persistence develops in *Chlamydia* and if persisting *Chlamydia* can be treated in vivo.

First, we tested the simultaneous application of MRY D2 and PEN G at an early development stage, which led to the formation of enlarged chlamydial cells, as seen for MRY D2 alone (Figure 6b and d). No synergistic effects were observed, which might be due to the fact that the effects of MRY D2 occurred upstream of the effects of PEN G activity in PGN biosynthesis. This result is similar to the activity of D-cycloserine. Recently, D-cycloserine was shown to target not only alanine racemases and Ddl but also transpeptidases in *B. subtilis* and *E. coli* due to their structural similarity with the native substrates [75]. For *Chlamydia*, inhibition of the alternative racemase GlyA and MurC/Ddl was shown in vitro [67,74] (Figure 8), and treatment of *C. trachomatis* with D-cycloserine also leads to the formation of enlarged chlamydial cells [29]. Simultaneous application of muraymycins and PEN G interferes with the same PGN synthesis sub-pathway as D-cycloserine, where the effect of inhibiting precursor synthesis outcompetes the inhibition of transpeptidation. However, treatment with D-cycloserine leads to ABs exhibiting nascent, non-cross-linked PGN instead of the lack of a PGN ring, with just a few remaining synthesis nodes of nascent non-cross-linked PGN, as seen with PEN G [38].

MRY D2 treatment of PEN G-induced persisting chlamydial cells cleared 70% of the infection in contrast to cells treated with the β-lactam only (Figure 6b,c). A reduced abundance of inclusions was also observed for MRY D2 treatment alone at the mid-stage of the developmental cycle in an active infection (Figure 6b,c). This indicates that while muraymycins had a bacteriostatic effect when applied early before the EB-to-RB differentiation and cell division, their later application exhibited bactericidal effects on actively dividing or persisting *C. trachomatis* cells.

One reason for the persistence-breaking bactericidal properties of MRY D2 could be the interference with another pathway besides PGN synthesis and/or a secondary target. Tunicamycin, another MraY inhibitor, additionally targets the glycosyltransferases TarO and CapM of the wall teichoic acid pathway and the 2-epimerases MnaA and Cap5P of the capsule synthesis in staphylococci [76,77]. Although these enzymes and MraY catalyze different reactions, they all use an UDP-activated hexose as a substrate. *Chlamydia* do not possess wall teichoic acid, a capsule, or an O-antigen; however, enzymes of other pathways with a similar substrate as MraY might be inhibited, which could then lead to the detrimental effect of the mid-stage application of muraymycins.

Another possible cause for this effect might be the timing itself. Normally, chlamydial cells differentiate into RBs and subsequently start cell division at ~8 hpi to 12 hpi [14,29,51,70]. During cell division, bacteria naturally shed PGN material into their surroundings; in *E. coli*, ~6% to 8% of their PGN is released per generation [78]. In *Chlamydia*, PGN is degraded by the lytic transglycosylase SpoIID, resulting in anhydro-Mur*N*Ac-tetra- or pentapeptides and the amidase AmiA, which releases the peptide chain from the sugar [79,80]. These PGN-derived peptides are subsequently recycled by the cytoplasmic peptidase YkfC by cleaving the γ-D-Glu-*m*DAP bond [81]. PGN fragments containing Mur*N*Ac-L-Ala-γ-D-Glu and L-Ala-γ-D-Glu-*m*DAP motifs are ligands for NOD1 and NOD2 receptors, respectively. These are located in the host cell cytoplasm and exhibit immune modulatory properties via NF-κB activation and the transcription of cytokines [82,83]. Application of muraymycins at the mid-stage of the chlamydial developmental cycle could lead to a complete block of new PGN synthesis that might be accompanied by continued PGN degradation. The antibiotic's interference with the pathway might lead to an increased shedding of chlamydial PGN, which could trigger the NOD-mediated host response in cervical HEp-2 cells and in cells of the female reproductive tract [84]. This NOD response could contribute to the clearance of chlamydial inclusions by increased inflammation and autophagy [85,86]. Although cell division is nearly completely blocked in PEN-induced persistent cells at 12 hpi [14], some PGN synthesis nodes remain [38]. Furthermore, continued shedding of NOD1 and NOD2 ligands was also reported for *C. trachomatis* upon treatment with different β-lactams [38,87,88]. An additional blockage of PGN precursor synthesis through muraymycins on top of the persistent state could then abolish the remaining PGN synthesis and intensify the cellular stress in these chlamydial cells. This might result in an enhanced NOD-mediated host response, eradicating *Chlamydia* from the host cells.

In contrast to cell culture infection models, *Chlamydia* infections in patients are not synchronized, and the pathogens are present at different stages of their developmental cycle, e.g., the early phase (before cell division started) and the mid-phase (active cell division). This could lead to mixed effects upon muraymycin treatment, inducing aberrance in the early phases and clearance in the mid phases. Cell culture infection models are also limited in reflecting the interplay of multiple stress-inducing factors like iron starvation, interferon-γ induced tryptophan starvation, or viral co-infections that may contribute to persisting phenotypes within infected human tissues and impact the antibiotic effect.

The results presented here support MraY as a promising target for the development of new antibiotic treatment strategies against *Chlamydia* and *Wolbachia*. However, to progress to selecting a muraymycin as a lead candidate for development, further medicinal chemistry is required to improve its potency and solubility, with a focus on penetrating multiple membrane barriers to reach intracellular pathogens and more in-depth analyses of toxicity, pharmacokinetic/pharmacodynamic (PK/PD) determination in appropriate in vivo models, and resistance development.

In conclusion, we showed that MRY D2 and its derivatives interfere with the activity of the transferase MraY in *Chlamydia* and *Wolbachia*. The structural conservation between the MraY of *Chlamydia* and *Wolbachia* and the MraY of *A. aeolicus* suggests that similar binding occurs in a competitive manner toward the natural substrate UDP-Mur*N*Ac. Treatment of *Wolbachia* with muraymycin derivatives resulted in the formation of enlarged bacterial cells, further strengthening the hypothesis that functional PGN synthesis is essential in these symbiotic bacteria of filarial nematodes. The effects of MRY D2 treatment on *Chlamydia* were dependent on the time of application. Based on these observations, we postulate the following: (i) in *Wolbachia* and nondividing *Chlamydia* in their early stage of the developmental cycle, inhibition of MraY-catalyzed lipid I formation has a bacteriostatic effect and induces the formation of enlarged, nondividing cells; (ii) the enlarged, aberrant cells lack PGN; and (iii) the interference of lipid I formation in actively dividing (mid-stage) or persisting *Chlamydia* abolishes (remaining) PGN synthesis and might also interfere with other bacterial or host cell mechanisms, resulting in a bactericidal effect on the *Chlamydia*.

The observed persistence-breaking property of MRY D2 could improve the understanding and treatment of reoccurring chlamydial infections in vivo.

4. Materials and Methods

4.1. Compounds

Muraymycin D2 and its derivatives MRH-22, -23, -25, -38, -76, -82, and -92 [47,48], the caprazamycin analog carbacaprazamycin [49], and DOX (Merck, Darmstadt, Germany) were dissolved in 100% (*v*/*v*) DMSO (Sigma-Aldrich, St. Louis, MO, USA).

4.2. Cell Culture and Bacterial Strains

Eukaryotic HEp-2 host cells (ATCC CCL-23, Cell lines service, Eppelheim, Germany) were cultured in Dulbeccos's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, CA, USA; item No. 31966021) supplemented with 10% (*v*/*v*) fetal calf serum (FCS; Gibco; item no. 10270106), 1 × MEM nonessential amino acids solution (Gibco), 1 × MEM vitamin solution (Gibco), 2.5 µg/mL amphotericin B (Gibco), and 50 µg/mL gentamicin (Gibco) at 37 °C and 5% (*v*/*v*) CO_2. Passaging of *C. trachomatis* D/UW-3/CX (ATCC VR-885) was performed every 2 to 3 days. Briefly, EBs were harvested by scraping off the host cell monolayer, vortexed with glass beads for 30 s, and centrifuged at 1480× *g* and 25 °C for 10 min. HEp-2 monolayers were inoculated with the EB suspension diluted in medium and incubated for 3 h. Then, the suspension was exchanged for fresh medium containing 1.2 µg/mL cycloheximide (Sigma-Aldrich). Cell cultures were tested monthly for *Mycoplasma* contamination using the Venor GeM OneStep Mycoplasma Detection Kit (Minerva Biolabs, Berlin, Germany). All cell culture experiments were conducted in supplemented growth DMEM medium without gentamicin, amphotericin B, and cycloheximide.

The insect cell line *A. albopictus* C6/36 (ATCC CRL-1660) was used to propagate *Wolbachia* endosymbionts (*Wolbachia* strain B of *A. albopictus*, as described in [32,33,89]. The cells were incubated in L15 Leibovitz's medium (Life Technologies, Carlsbad, CA, USA) supplemented with 5% (*v*/*v*) FCS (PAA Laboratories, Cölbe, Germany), 1% (*v*/*v*) nonessential amino acids (PAA Laboratories), 2% (*w*/*v*) tryptose phosphate broth (Sigma-Aldrich), and 1% (*v*/*v*) penicillin/streptomycin (PAA Laboratories, Cölbe, Germany) at 26 °C.

4.3. Cytotoxicity of Compounds toward HEp-2 Cells

Cytotoxicity of the compounds toward HEp-2 cells was assessed using the resazurin-based alamarBlue cell viability reagent (Thermo Fisher Scientific, Waltham, MA, USA). 5×10^4 HEp-2 cells/mL were seeded into flat base TC-96-well plates (Sarstedt, Nümbrecht, Germany) and incubated at 37 °C and 5% (*v*/*v*) CO_2. After 48 h, the cells were washed with medium, followed by the addition of compounds in a serial dilution ranging from 128 µg/mL to 1 µg/mL for another 28 h. Next, the cells were washed twice with Hank's balanced salt solution (HBSS) and incubated with the alamarBlue cell viability reagent diluted 1:10 in HBSS for 1 h. The conversion of resazurin into resorufin by viable cells was determined by transferring the supernatant into black 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and measuring the fluorescence at 550 nm excitation and 595 nm emission wavelengths using a Tecan infinite M200 plate reader and Tecan SparkControl software v3.2 (Tecan Group, Männedorf, Switzerland). HEp-2 cell viability was normalized to the vehicle control, and the cytotoxic concentration 50% (CC_{50}) was calculated by non-linear regression using GraphPad Prism v10.1.2 (GraphPad Software, San Diego, CA, USA).

4.4. Effect of Compounds on Active and Penicillin G-Induced Persistent C. trachomatis Infections

The effect of the compounds on active *C. trachomatis* infections was analyzed by fluorescence microscopy. First, 3.5×10^4 HEp-2 cells/mL were seeded into black 96-well plates (ibidi, Gräfeling, Germany) and incubated for 3 days at 37 °C and 5% CO_2. The cells were washed once with medium and infected with freshly prepared *C. trachomatis* D/UW-3/CX EB-containing suspension. After 2 h incubation, the EB suspension was aspirated, and the cells were washed twice with medium. The effect of compounds on an infection

was determined by application of the compound at 2 hpi or 12 hpi in a serial dilution from 1 µg/mL to 64 µg/mL (MRH-25, -76, -92, cCPZ) or from 1 µg/mL to 128 µg/mL (MRY D2, MRH-22, -23, -38, and -82). Negative controls were conducted with DMSO as a vehicle control. For *Chlamydia*, the MIC is defined as a >90% reduction in inclusions [52]. The MIC of compounds inducing the formation of enlarged, aberrant bodies is the lowest concentration that induces the abnormal inclusion morphology [39].

The effect of MRY D2 on penicillin-induced persistent *C. trachomatis* infections was analyzed as described for the active infection, with the following modifications. After removal of the EB-containing lysate at 2 hpi and washing of the cells, 100 U/mL PEN G was added to induce a persistent infection. MRY D2 was added at 12 hpi in a concentration range of 128 µg/mL to 1 µg/mL by serial dilution. In another approach, MRY D2 was applied simultaneously with PEN G at 2 hpi to determine if early administration influences the establishment of a persistent infection.

Infected HEp-2 cells were fixed and permeabilized with 100% (*v*/*v*) ice-cold methanol at 30 hpi and washed once with 1 × phosphate-buffered saline (PBS, 4 mM KH_2PO_4 pH 7.4, 16 mM Na_2HPO_4, 115 mM NaCl). The Pathfinder Chlamydia Culture Confirmation System (Bio-Rad Laboratories, Hercules, CA) was used to stain the *C. trachomatis* lipopolysaccharide and host cell cytoplasm by incubation for 30 min at 37 °C. Genomic DNA was stained with 10 µg/mL DAPI (Thermo Fisher Scientific) for 1 min and then washed twice with PBS for 10 min at 4 °C. Fluorescence microscopy was performed with an Axio observer Z.1 using the Zen2 software (Carl Zeiss, Oberkochen, Germany).

Inclusions were quantified using CellProfiler 4.0.6. Cell image analysis software (Borad Institute, Cambridge, MA, USA; [90]) was used to identify fluorescein-labeled *Chlamydia* clusters as the primary objects. The chlamydial cell phenotype in each inclusion was assessed manually and categorized by inclusions filled with (i) EBs and/or RBs, (ii) enlarged, aberrant bodies similar to the penicillin G-induced ABs, and (iii) a mixed phenotype of inclusions filled with EBs/RBs and AB (typically just one). Statistical analysis for all parameters was performed with an unpaired, two-tailed Student's *t*-test against the respective vehicle control using GraphPad Prism software version 10.1.2 for Windows (GraphPad Software).

4.5. Fluorescence Microscopy of Muraymycin-Treated Wolbachia

The effect of muraymycin derivatives on the phenotype of *Wolbachia* was analyzed by fluorescence microscopy as described [33], with minor modifications. Briefly, 1.5×10^4 *A. albopictus* C6/36 cells infected with *Wolbachia* were seeded into 8-well culture slides and incubated with or without compound at 26 °C. Medium was exchanged every third day, with and without compound. The cells were fixed on day 9 using 4% (*w*/*v*) paraformaldehyde in PBS. The cells were permeabilized with 0.25% (*v*/*v*) Triton X-100 and washed with PBS supplemented with 2% (*w*/*v*) bovine serum albumin (BSA Fraction V, Fisher Scientific, Schwerte, Germany) and 0.1% (*v*/*v*) Triton X-100 (PBST). Genomic DNA was stained with 0.25 µg/mL DAPI (Sigma-Aldrich), and the cells were washed with PBST. Slides were embedded in Vectashield Mounting Medium (Vector Laboratories, Newark, CA, USA), and microscopy was performed with a Leica fluorescence microscope DM RD (Leica Camera, Wetzlar, Germany). Photoshop CS2 software (Adobe Systems, San Jose, CA, USA) was used for editing the sharpness, brightness, and contrast of the images. ZEN 3.5 (blue edition) software (Zeiss, Oberkochen, Germany) was used in combination with the image analysis module to determine the diameter of *Wolbachia*. Enlarged cells were defined as having a diameter greater than the 75th percentile of the DMSO control. For statistical analysis, a Kruskal–Wallis test was performed using GraphPad Prism software version 10.1.2 for Windows (GraphPad Software).

4.6. Quantification of Wolbachia

Susceptibility of *Wolbachia* toward different compounds was tested as described [32,53]. In short, 1×10^4 cells/well of *A. albopictus* C6/36 infected with W*olbachia* were cultured in supplemented medium in 96-well plates with or without compound at 26 °C for 9 days. The medium, with and without compound, was exchanged every third day, and the cells were harvested on day 9 for the extraction of genomic DNA using the QIAamp Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. *Wolbachia* were quantified by qPCR of the *16S rDNA* gene of *Wolbachia* (GenBank accession No. X61767) and the *actin B* gene of *A. albopictus* (GenBank accession no. DQ657949) as described [32,91]. A plasmid containing the appropriate insert was used to generate a standard curve to determine the copy number of the genes. The abundance of *Wolbachia* was normalized by calculating the ratio of *16S rDNA* copies/µL to *actin B* copies/µL.

4.7. Purification of C. pneumoniae MraY

The heterologous transmembrane protein *C. pneumoniae* MraY ($MraY_{Cpn}$, primary accession No. A0A0F7WR30) was produced as described in Henrichfreise et al. (2009) [32], with minor changes. Briefly, the plasmid pET20+$mraY_{Cpn}$ was transformed into *E. coli* C43(DE3) [92], and expression of transcription was induced in 4 L 2 × YT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) by 1 mM IPTG (Thermo Fisher Scientific) at an OD_{600nm} of 0.6 with shaking at 25 °C for 16 h. Harvested cells were washed in 200 mL of 25 mM Tris-HCl (pH 8) and resuspended in buffer A (25 mM Tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 150 mM NaCl, 30% (*v*/*v*) glycerol, 1 mM $MgCl_2$). After adding 1 U/mL benzonase (Sigma-Aldrich) and increasing the $MgCl_2$ concentration to 3 mM, the cells were sonicated and centrifuged at 31.85× *g* at 4 °C for 30 min. The pellet was resuspended in 10 mL buffer A supplemented with 17.8 mM n-dodecyl-β-D-maltoside (DDM, Carl Roth, Karlsruhe, Germany) and incubated for 50 min at 4 °C. The supernatant was separated from membrane debris by centrifugation, and the obtained pellet was solubilized a second time with 21.5 mM DDM. Each supernatant containing $MraY_{Cpn}$ was then incubated with 1.5 mL Ni-NTA agarose (Macherey-Nagel, Düren, Germany) and equilibrated twice with 10 mL buffer A under gentle shaking at 4 °C for 3 h. The mixtures were poured onto affinity chromatography columns and rinsed with 7.2 mL buffer A followed by 7.2 mL washing buffer (buffer A supplemented with 3.9 mM DDM, 10 mM imidazole). $MraY_{Cpn}$ was eluted by the sequential addition of 3 × 0.5 mL elution buffer (buffer A with 3.9 mM DDM) containing 100 mM, 200 mM, and 300 mM imidazole, respectively. Eluates were stored at −70 °C.

4.8. Inhibition of C. pneumoniae MraY In Vitro

Inhibition assays with $MraY_{Cpn}$ and the compounds were performed based on the activity assays for $MraY_{Cpn}$ as previously described [32], with minor modifications, in a final volume of 50 µL. First, 2.5 nmol C_{55}-P (Larodan, Solna, Sweden) was dissolved in 0.43% (*v*/*v*) Triton X-100 by vortexing for 1 min. Then, 75 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 10% (*v*/*v*) DMSO, and UDP-Mur*N*Ac-pentapeptide obtained from *Bacillus cereus* DSM2302 (based on [93]) were added. Compounds were applied at the indicated concentrations and incubated with 2 µg $MraY_{Cpn}$ at 30 °C for 90 min. The reaction products were extracted with 50 µL *n*-butanol-pyridine acetate (2:1, pH 4.2) by vortexing for 1 min. After centrifugation at 17,000× *g* for 5 min, components of the organic phase were separated by thin layer chromatography (TLC) on HPTLC alumina silica gel 60 plates (Merck) using chloroform–methanol–water–ammonia (88:48:10:1) as a mobile phase [94]. The TLC plates were stained with phosphomolybdic acid stain (2.5% (*w*/*v*) phosphomolybdic acid, 1% (*w*/*v*) ceric-sulphate, 6% (*v*/*v*) sulfuric acid) and visualized at 120 °C. The experiments were performed in duplicate.

4.9. In Silico Analysis of MraY

The MraY protein sequence of *A. aeolicus* VF5 (primary accession No. O66465), *C. trachomatis* D/UW-3/CX (primary accession No. O84762), *C. pneumoniae* GiD (primary accession No. A0A0F7WR30), and *Wolbachia* endosymbionts of *A. albopictus* (primary accession No. A0A4S2QUK2) and *B. malayi* (primary accession No. Q5GRZ3) were analyzed in silico. Multiple alignments were performed with Clustal Omega Software v1.2.4 (EMBL-EBI, Hinxton, UK). Predictions of $MraY_{Ctr/Cpn/wAlbB/wBm}$ protein models were made with one-to-one threading against the crystal structure of the $apoMraY_{Aae}$ chain A (PDB ID: 4J72.A, [58]) or of $MraY_{Aae}$ bound to MRY D2 (PDB ID: 5CKR, [50]) using phyre2 software (Imperial College London, London, UK; [95]) and visualized with ChimeraX (UCSF, San Francisco, CA, USA; [96]). Residues of the $MraY_{Aae}$ active site or residues contributing to the binding of muraymycin D2 or carbacaprazamycin were indicated in the multiple alignment and protein models based on [54–58].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13050421/s1, Table S1: Cytotoxic concentration 50% (CC_{50}) of muraymycin (MRY) D2, its derivatives (MRHs), and carbacaprazamycin (cCPZ) on eukaryotic HEp-2 cells, Figure S1: Chemical structures of muraymycin derivatives and carbacaprazamycin, Figure S2: Most muraymycin derivatives induce the formation of enlarged chlamydial cells, Table S2: Sequence identities and confidence of *C. trachomatis*, *C. pneumoniae*, and *Wolbachia* endosymbionts of *A. albopictus* or *B. malayi* MraY protein models against *A. aeolicus* apoMraY or $MraY_{Aae}$ bound to muraymycin D2.

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Letter to the Editor

No resistance development against corallopyronin A in *Wolbachia* in C6/36 cell culture

Editor: D. Leitsch

Dear Editor,

Corallopyronin A (CorA) is a myxobacterial (*Corallococcus coralloides*) natural product inhibitor of bacterial DNA-dependent RNA polymerase (RNAP) being developed to treat filarial infections [1,2]. Due to its distinct binding site in the RNAP switch region, there is no cross-resistance with rifampicin, another RNAP inhibitor [3].

CorA is effective against Gram-positive bacteria and intracellular Gram-negative bacteria, e.g., *Wolbachia spp.* endosymbionts [2]. CorA-resistant mutants have been found for *Staphylococcus aureus* [4] and *Neisseria gonorrhoeae* [5]. Mutations conferring CorA resistance in *Staphylococcus aureus* arise at a 4.5-fold lower frequency than for rifampicin [4]. However, for *N. gonorrhoeae,* high-level CorA resistance could only be achieved via a multi-step selection process requiring a mutation in an efflux pump. When selecting at the standard 4 × MIC, mutants could not be recovered, indicating a low frequency of mutation to resistance of $\leq 10^{-10}$ [5]. To date, anti-wolbachial *in vitro* and *in vivo* experiments have not indicated resistance to CorA. However, as bacteria inevitably develop resistance to antibiotics over time, we investigated the potential for resistance development against CorA in the *Wolbachia pipientis* supergroup B strain of *Aedes albopictus* (*w*AlbB) infecting *Aedes albopictus* C6/36 cells under long-term treatment conditions. A high frequency of mutation to resistance would raise concerns about the long-term efficacy of CorA and could limit its clinical development, especially given the extended treatment durations typically required for effective targeting of *Wolbachia* [1].

As obligate intracellular bacteria, a standard determination of the minimal inhibitory concentration (MIC) cannot be performed for *Wolbachia*. Therefore, we defined the MIC as the lowest concentration of an antimicrobial that will lead to a *16S rRNA*/*act* ratio <0.5. We performed a 9-day assay with different CorA concentrations as published [1]. DNA was extracted to monitor *Wolbachia* numbers using *16S rRNA* (a single copy gene) qPCR normalised to the *A. albopictus* actin gene *act* and the *16S rRNA*/*act* ratios were plotted [1]. This resulted in an MIC of 31.25 nM CorA (Fig. 1A).

Cultures with 6.87×10^8 *Wolbachia*/well (200 µL) were treated in quadruplicate (2.75×10^9 *Wolbachia*) with either an optimal concentration of 125 nM (4 × MIC) or suboptimal concentrations of 31.25 nM (1 × MIC) and 15.63 nM (0.5 × MIC) CorA (batch № R545, purity 91.4%) or 0.25% DMSO (vehicle control) for up to 245 days [1]. Medium with or without CorA was changed every second day and cells were split 1:5 every seven days. The suboptimal concentration of 31.25 nM CorA was used to allow for resistance to develop. After 140 and 217 days, these cells were then challenged with a high concentration of 125 nM. DNA was extracted every seven days and the *16S rRNA*/*act* ratio calculated [1]. *Act* copies, and hence C6/36 cell numbers, were constant (mean ± SEM: control = $1.48 \times 10^8 \pm 1.05 \times 10^7$ copies/well, treated = $2.31 \times 10^8 \pm 0.56 \times 10^7$ copies/well).

From day 0 to day 7, all wells had a drop in *Wolbachia* that was higher (up to 1-log) in the presence of CorA (Fig. 1B). Except for the 125 nM CorA-treated group, *Wolbachia* numbers increased until day 35 and then stabilised. Treatment with 31.25 nM (pink circle) resulted in a mean 1-log reduction. Increasing the CorA concentration from 31.25 nM to 125 nM after 140 (brick red circle) or 217 (orange circle) days, resulted in *Wolbachia* depletion >3-logs (brick red circle), reaching the level of the 125 nM-treated group, or to an almost 2-log drop (orange circle) until the experiment was terminated. Returning the CorA concentration back to 31.25 nM (purple circle, dotted line) on day 217 resulted in an increase of *Wolbachia*. Treatment with 125 nM CorA (blue) depleted *Wolbachia* by mean 4.9-logs between days 28 and 245. When CorA was removed after 35 days (open blue square), the *Wolbachia* numbers appeared to increase slightly, but the increase was in the range of the change seen in the 125 nM treatment over the 245-day experiment (4.14×10^{-5} (D217) to 2.92×10^{-3} (D126)). Treating these cells again with 125 nM from D70 (dark blue square, dotted line) depleted *Wolbachia* to the same level as before, indicating that they were still sensitive.

No loss of sensitivity in *Wolbachia* towards CorA was observed and hence no resistance was selected. From our results we predict a low frequency of mutation to resistance of $<2.75 \times 10^{-9}$, similar to the frequency predicted for *N. gonorrhoeae* ($\leq 10^{-10}$) and lower than the 1.48×10^{-8} determined for *S. aureus* [4,5]. Thus, with proper use, resistance to CorA in *Wolbachia* should be slow to develop, removing this as a hurdle for its continued preclinical and clinical development.

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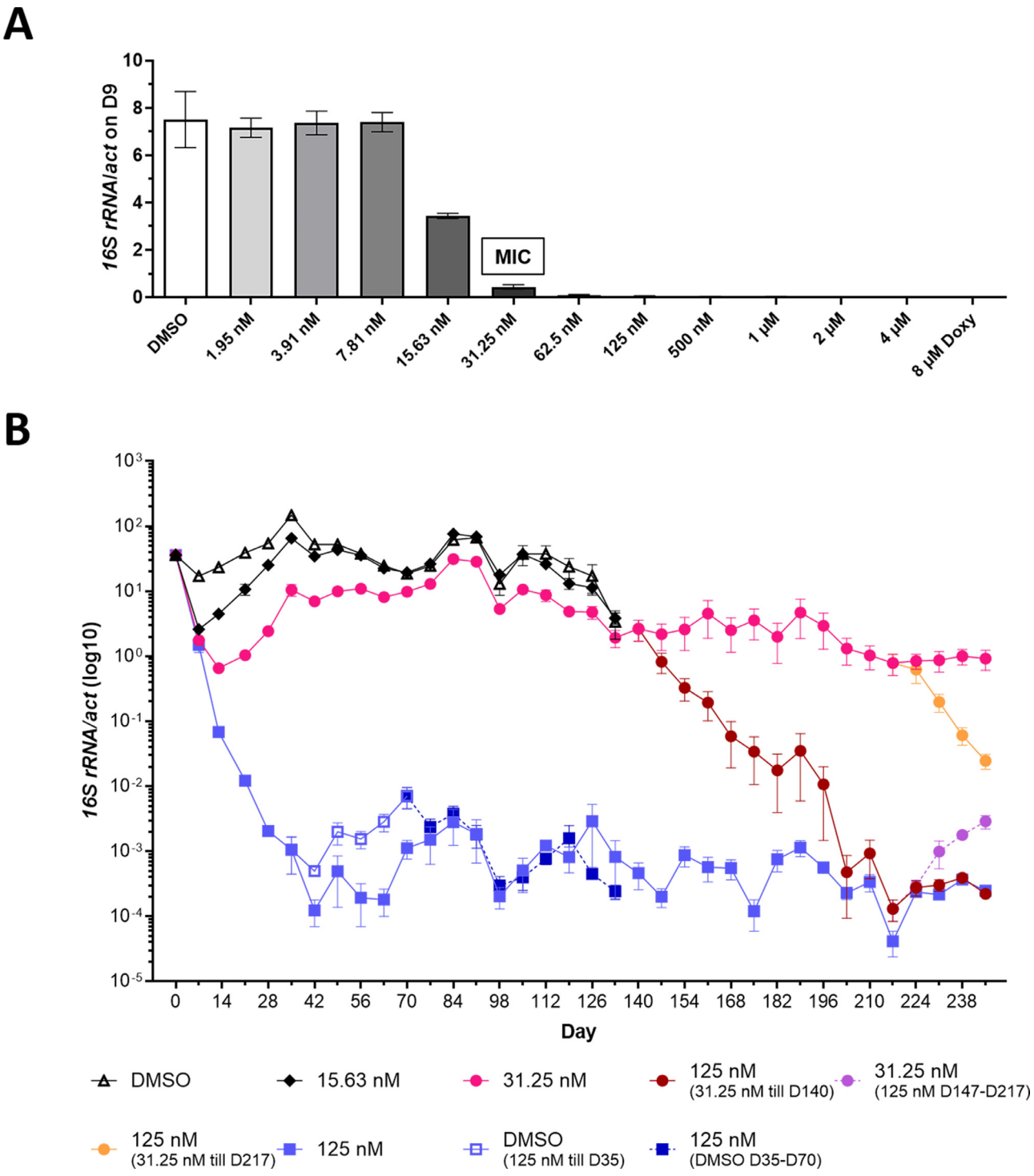

Fig. 1. Long-term CorA treatment of *Wolbachia* in C6/36 cells. (A) To determine the minimal inhibitory concentration (MIC), *Wolbachia* were treated with different concentrations of CorA for 9 days, DMSO as a negative control, or doxycycline (Doxy) as a positive control. (B) *Wolbachia* were treated with 125 nM, 31.25 nM or 15.63 nM CorA, or DMSO for 245 days. The 125 nM-treated group (blue square) was split on D35 and cultured without CorA (open blue square) until D70, after which CorA treatment was restarted (dark blue square, dotted line). The 31.25 nM-treated group (pink circle) was split on D140 and cultured with 125 nM CorA (brick red circle). The new 125 nM-treated group was returned to 31.25 nM on D217 (purple circle, dotted line). The 31.25 nM was split into 31.25 nM and 125 nM (orange circle) a second time on D217. The DMSO (open black triangle) and 15.63 nM-treated groups (black rhombus) were stopped on D133. Numbers in parentheses in the legend indicate the previous CorA concentration and treatment day when CorA was decreased or increased. DNA was extracted on (A) day 9 or (B) every seven days and the 16S rRNA and actin genes quantified by qPCR. The *16S rRNA*/*act* ratios (mean ± SEM of four replicates) were plotted with GraphPad Prism 10.

CRediT authorship contribution statement

Lara Vanessa Behrmann: Formal analysis, Investigation, Visualization, Writing – original draft. **Christine Lämmer:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Andrea Schiefer:** Conceptualization, Writing – review & editing. **Helene Neufeld:** Investigation, Writing – review & editing. **Miriam Grosse:** Resources, Writing – review & editing. **Marc Stadler:** Resources, Supervision, Writing – review & editing, Funding acquisition. **Gabriele Bierbaum:** Formal analysis, Writing – original draft. **Achim Hoerauf:** Supervision, Writing – review & editing, Funding acquisition. **Kenneth Pfarr:** Conceptualization, Supervision, Writing – original draft, Funding acquisition.

Declarations

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Declaration of competing interests: KP, AS and AH hold patents for CorA: US 9168244 B2, US 9687470 B2, EP 2704708 B1 and a European patent-pending EP 20 172 409.3. All other authors declare no conflict of interest.

Ethical approval: Not required.

Sequence information: Not applicable.

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