University of Alberta

Horizontal transfer of genes and genomes in an endosymbiotic system: Investigation of the movement of intracellular bacteria and related gene transfer

by

Philip Daniel Batista

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Physiology, Cell and Developmental Biology

Department of Biological Sciences

© Philip Daniel Batista
Fall 2013
Edmonton, Alberta

Permission is hereby granted to the University of Alberta Libraries to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only. Where the thesis is converted to, or otherwise made available in digital form, the University of Alberta will advise potential users of the thesis of these terms.

The author reserves all other publication and other rights in association with the copyright in the thesis and, except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatsoever without the author's prior written permission.

Abstract

The genus *Wolbachia* is comprised of intracellular gram negative bacteria capable of infecting a wide range of insect hosts. In arthropods, *Wolbachia* are reproductive parasites that can cause cytoplasmic incompatibility, parthenogenesis, feminization or male killing, each of which enhances the vertical transmission of the endosymbiont in a host population. Horizontal transmission of *Wolbachia* between different species can be shown by incongruence of phylogenies of *Wolbachia* and their hosts along with *Wolbachia's* widespread distribution. *Wolbachia* is a model for understanding the horizontal transfer of genes and genomes and the role of mobile genetic elements, including bacteriophages in host-symbiont interactions. My study provides the groundwork for understanding multitrophic interactions among insects, bacteria, and viruses in *Wolbachia*-host systems.

This study examines horizontal transfer of *Wolbachia* and associated WO bacteriophages in two host-parasitoid systems based on agronomically important pests *Plutella xylostella* and *Ceutorhynchus obstrictus.* Phylogenetic analysis shows that *Wolbachia* and WO have been horizontally transferred between *P. xylostella* and the parasitoid *Diadegma insulare* but not between *C. obstrictus* and its parasitoid *T. lucidus*. Results from this study suggest that horizontal transfer of *Wolbachia* requires longer-term host/parasitoid associations.

Comparative genomic methods were used to identify the core genome of the replicating bacteriophage WO. This study shows that all replicating WO bacteriophages include modules for DNA packaging and head assembly and tail

morphogenesis. Remnant prophages lack the tail morphogenesis module and many genes associated with DNA packaging. One bacteriophage gene implicated in the establishment and maintenance of *Wolbachia* symbiosis is a DNA adenine methyltransferase (MTase) containing a ParB-like nuclease domain. Adenine methylation of DNA in bacteria has numerous functions including gene regulation and host-pathogen interactions. This study reveals that this gene is widespread in prokaryotes and has undergone horizontal transfer between distantly related bacterial species, but not between *Wolbachia* strains. Examining the genomic position of this gene in each prokaryotic genome revealed a conserved association with bacteriophage DNA packaging genes.

Acknowlegements

I would like to express my sincere appreciation to my supervisors Dr. Harriet Harris, for giving me the opportunity to work in your lab and introducing me to world of *Wolbachia* research, and Dr. Andrew Keddie for your advice and critical guidance. I am grateful for two excellent supervisors who have given me their time and knowledge throughout the years. It is because of your support and constant encouragement that this thesis is possible.

I would like to thank Dr. Jocelyn Hall, Dr. Maya L. Evenden and Dr. Alberto Severini for being part of my defense and for their insightful and constructive suggestions about my work. Special thanks to Dr. Lloyd Dosdall for being a part of my candidacy exam and for his help with the indentification of the parasitoid species used in this thesis.

Many thanks to my distinguished colleagues that I have had the pleasure to collaborate with - Dr. Lesley Brennan and Dr. Jennifer Haukedal. I am grateful for the endless discussions and shared fustrations of *Wolbachia* research that we have had over the years. It has been a pleasure to work with you both. Thank you to the undergraduate students who I have had the opportunity to work with and have participated in my research: Kailey Michal, Mark Franklin, Maxwell Harrison, and Dylan Chase.

Lastly I would like to thank all my friends and family for their constant love and support throughout the long PhD process. I am proud to have you all in my life and this work is dedicated to you.

Table of Contents

Chapter 1: Introduction……..…….1

Wolbachia and endosymbiotic bacteria ……...……...……..1

Cell biology and reproductive phenotypes……..……..2

Fitness effects of *Wolbachia*……...……...……7

Wolbachia phylogeny……..10

Wolbachia infection in agricultural pests……12

Genome characteristics……15

Endosymbiotic phages – role in symbiont biology……...……..….16

Objectives……....18

Figure 1.1……..…20

References …….……..22

Chapter 2: Horizontal transfer of *Wolbachia* in host-parasitoid systems

2A: *Plutella xylostella and Diadegma insulare*

Introduction……..34

Materials and methods…….37

Results……..40

Discussion……41

2B: *Ceutorhynchus obstrictus* and associated parasitoids

Introduction……..45

Materials and methods…….49

Results……..53

Discussion……54

Table 2.1……..60

Table 2.2……..60

Figure 2.1……61

Figure 2.2……62

Figure 2.3……63

Figure 2.4……64

Figure 2.5……65

Figure 2.6……66

Figure 2.7……68

References ……..……..69

Chapter 3: Horizontal transfer of phages between *Wolbachia*

Introduction……..77

Materials and methods……82

Results……..83

Discussion……88

Figure 3.1……93

Figure 3.2……94

Figure 3.3……95

Figure 3.4……96

References …….....97

Chapter 4: Molecular cloning and characterization of the ParB-methyltransferase in *Wolbachia*

Introduction……103

Materials and methods……..109

Results……116

Discussion……..121

Table 4.1……....129

Table 4.2……130

Figure 4.1……..134

Figure 4.2……..135

Figure 4.3 ……136

Figure 4.4…….137

Figure 4.5……..138

Figure 4.6……..140

Figure 4.7……..141

Figure 4.8……..142

Figure 4.9……..143

Figure 4.10……144

Figure 4.11……146

References ……147

Chapter 5: Conclusions……159

Appendix I: Phylogenetic Analysis……..161

List of Tables

Table 2.1. Primers used for PCR amplification of different *Wolbachia* genes used in this study.

Table 2.2. Primers used for qPCR amplification

Table 4.1: BP Cloning Primers

Table 4.2: ParB-MTase containing genomes used in this study

List of Figures

Figure 1.1. Phylogeny of *Wolbachia* within the order Rickettsiales.

Figure 2.1. Rearing cage setup for rearing of *Plutella xylostella* and *D.insulare.*

Figure 2.2. Phylogenetic tree of *Wolbachia* found in *Plutella xylostella* and *Diadegma insulare* based on the concatenated alignment of *groEL* and *ftsZ.*

Figure 2.3. Phylogenetic tree of bacteriophage WO found in P*lutella xylostella* and *Diadegma insulare* based on *orf7.*

Figure 2.4. Maximum-likelihood phylogeny of *Wolbachia* in different populations of *Plutella xylostella* based on *wsp.*

Figure 2.5. Phylogenetic tree of *Wolbachia* found in *Ceutorhynchus obstrictus* and *Trichomalus lucidus* based on the concatenated alignment of gatB and ftsZ.

Figure 2.6. Phylogenetic tree of *Wolbachia* found in *Ceutorhynchus obstrictus* and *Trichomalus lucidus* based on the concatenated alignment of orf7.

Figure 2.7. Comparison of larval weight and *Wolbachia* copy number in CSW from *B. napus* and *B. rapa*.

Figure 3.1. Protein map of the conserved core DNA packaging and head assembly module and tail morphogenesis module of WORiC.

Figure 3.2. Whole genome comparisons between WORiC, WOCauB2, WOVitA1, WOMelB, and WORiB.

Figure 3.3. Whole genome comparisons between WORiC, WOMelA, WORiA, and WORiB.

Figure 3.4. Phylogeny of terminase and baseplate assembly protein W amino acid sequences.

Figure 4.1. Schematic of primer design for the amplification of seperate ParB and MTase domains for Gateway cloning.

Figure 4.2. Schematic of gateway cloning of ParB and MTase domains.

Figure 4.3. Amino acid alignment of Met1 and Met2 proteins of *Wolbachia* strain wMel.

Figure 4.4. Phylogenetic tree of *Wolbachia* based on met gene sequences.

Figure 4.5 Phylogenetic tree of 16S organismal phylogeny of alphaproteobacteria and of associated ParB-MTase.

Figure 4.6 Phylogenetic tree of ParB-MTase found in different bacteria.

Figure 4.7: Gene neighborhood of ParB-MTase in Firmicutes (I).

Figure 4.8: Gene neighborhood of ParB-MTase in Proteobacteria clade (II).

Figure 4.9: Gene neighborhood of ParB-MTase in Proteobacteria clade (III).

Figure 4.10: Phylogenetic tree based on (A) Terminase (B) Portal Protein and (C) ParB-MTase.

Figure 4.11: Growth curves of the ParB domain and MTase domain from *Wolbachia* wRi expressed in *E.coli*.

List of Abbreviations

5mC	C5-methylcystosine
AdoMet	S-adenosyl-$_L$-Methionine
AIC	Akaike information criterion
AU	approximately unbiased test
CcrM	cell-cycle regulated DNA MTase
CI	Cytoplasmic Incompatability
CSW	Cabbage Seedpod Weevil
Dam	DNA adenine methyltransferase
DBM	Diamondback Moth
ef1a	Elongation factor 1 α
ftsZ	cell division protein
gatB	Glutamyl-tRNA (Gln) amidotransferase, subunit B
groEl	heat-shock protein HSP60
GTR	General Time Reversible model
HGT	horizontal gene transfer
ML	Maximum likelihood
mod	modification factor
MP	Maximum parsimony
MTases	DNA methyltransferases
N4mC	N4-methylcytosine
N6mA	N6-methyladenine
orf7	WO phage minor capsid protein

ORFs	open reading frames
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
R-M	Restriction-Modification
resc	rescue factor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH	Shimodaira-Hasegawa test
SIT	Sterile insect technique
SNBP	Sperm Nuclear Basic Proteins
STE buffer	Sodium Chloride-Tris-EDTA
TBR	tree bisection and reconnection
TEV	Tobacco Etch Virus
TRD	target recognition domain
TVM	Transversion model
wsp	*Wolbachia* surface protein
wBm	*Wolbachia* endosymbiont of *Brugia malayi*
*w*CauA	*Wolbachia* endosymbiont of *Cadra cautella* (A-supergroup)
*w*CauB	*Wolbachia* endosymbiont of *Cadra cautella* (B-supergroup)
*w*Cep	*Wolbachia* endosymbiont of *Ostrinia scapulali*
*w*Cof	*Wolbachia* endosymbiont of *Corcyra cepharonica*
wCon	*Wolbachia* endosymbiont of *Tribolium confusum*
*w*Kue	*Wolbachia* endosymbiont of *Ephestia kuehniella* (Yokohama)

wMel	*Wolbachia* endosymbiont of *Drosophila melanogaster*
wPip	*Wolbachia* endosymbiont of *Culex pipiens*
wRi	*Wolbachia* endosymbiont of *Drosophila simulans* Riverside
wSus	*Wolbachia* endosymbiont of *Plutella xylostella*
*w*Sca	*Wolbachia* endosymbiont of *Ephestia kuehniella* (Tsuchiura)
*w*Tai	*Wolbachia* endosymbiont of *Teleogryllus taiwanemma*
*w*VitA	*Wolbachia* endosymbiont of *Nasonia vitripennis* (A-supergroup)
*w*VitB	*Wolbachia* endosymbiont of *Nasonia vitripennis* (B-supergroup)
wXyl	*Wolbachia* endosymbiont of *Plutella xylostella*

Chapter 1

Introduction

Wolbachia and endosymbiotic bacteria

Our current understanding of the interactions between arthropods and heritable intracellular bacteria has been greatly enhanced by a wide range of techniques that generate molecular information. These techniques have provided insight into the roles that intracellular bacteria have in the biology of their host. Arthropods harbour numerous types of heritable endosymbiotic microbes including bacteria, both as obligate primary and facultative secondary endosymbionts, and a substantial number of ectosymbiotic bacteria (Gosables et al., 2010). Although primary endosymbionts in arthropods are essential to the development and reproduction of hosts, facultative endosymbionts are not. Unlike primary symbionts which have coevolved with their hosts, facultative endosymbionts are capable of being transferred to new host species. The acquisition of facultative endosymbionts increases the genetic repertoire of their host, providing a host with new capabilities such as nutrient acquisition, or resistance to parasites or pathogens. Unlike primary endosymbionts, which satisfy a specific role within their hosts, facultative endosymbionts are capable of acquiring new genes through horizontal gene transfer.

Hertig and Wolbach (1924) initially identified Gram negative intracellular bacteria in *Culex pipiens* (L.) (Diptera: Culicidae) while screening mosquitoes for rickettsia. These bacteria were present in germ cells of *C. pipiens* during all stages

of development. Based on this work Hertig and Wolbach (1924) suggested vertical transmission from mother to offspring to explain the high infection rates in these insects. Later Hertig (1936) used the name *Wolbachia pipientis* for this organism, although the symbiotic relationship between *Wolbachia* and its host was unknown at the time. In 1971, Yen and Barr showed that *Wolbachia* modify host reproduction in incompatible crosses between different *C. pipiens* populations. Removing the *Wolbachia* infection by antibiotic treatment restored compatiblility between the same populations of *C. pipiens* (Yen and Barr, 1973). The ability of *Wolbachia* to manipulate host reproduction has enabled these bacteria to become the most widespread endosymbiont, estimated to infect up to 66% of all insect species (Hilgenboecker et al., 2008).

Cell biology and reproductive phenotypes

Wolbachia was initially identified in the germ cells of *C. pipiens* (Hertig and Wolbach, 1924). It has more recently been observed in numerous tissues, including haemolymph, fat body, Malphigian tubules, and nervous tissue (Dobson et al., 1999; McGraw and O'Neill, 2004; Mercot and Charlat, 2004) in diverse insect species. *Wolbachia* is considered a reproductive parasite in arthropods because it is known to manipulate host reproduction to enhance its own vertical transmission. In different hosts *Wolbachia* induces cytoplasmic incompatibility (Yen and Barr, 1973), parthenogenesis (Stouthamer et al., 1993), feminization (Rigaud et al., 1991) or male killing (Jiggins et al., 1998). The molecular mechanisms behind these manipulations have not been determined.

Cytoplasmic incompatibility (or CI) leads to non-viable progeny in crosses between uninfected females and *Wolbachia*-infected males. CI occurs during the first mitotic division following fertilization due to the inability of the paternal chromosomes to condense and segregate at the same rate as the maternal chromosomes (reviewed in Harris and Braig, 2003; Tram et al., 2003). Since *Wolbachia* is not present in mature sperm, it is thought that *Wolbachia* must modify the sperm nuclei in some way during spermatogenesis (Masui et al., 2000; Tram et al., 2003). The result of this modification is seen after fertilization in an uninfected embryo, where defects in the replication-independent nucleosome assembly, indicated by delays in histone H3.3 incorportation on to paternal chromatin after protamine removal, explains the observed improper chromatin condensation and defects in DNA segregation in incompatible crosses (Landman et al., 2009).

One model describing CI is the "lock and key model", suggesting a distinct modification factor (*mod*) in sperm and a rescue factor (*resc*) in eggs (Poinsot et al., 2003). In this model these factors are controlled by different *Wolbachia* genes. When more than one *Wolbachia* strain is present (double or triple infections) a bidirectional CI may occur. Bidirectional CI, in populations of *Drosophila simulans* (Sturtevant) (Diptera: Drosophilidae), and many other insects, occurs when sperm from a male infected with a *Wolbachia* strain capable of inducing CI fertilizes an egg that is infected with a different *Wolbachia* strain (O'Neill and Karr, 1990). Here the *mod* on the paternal chromosomes is incompatible with the rescue factor found in the egg.

CI can be avoided when an egg infected with two CI inducing *Wolbachia* strains is fertilized with sperm from males with either, or both of the *Wolbachia* strains. In two lines of *D. simulans* each containing a different strain of CI inducing *Wolbachia*, crosses between males and females from the two lines result in a bidirectional incompatibility. However, females infected with both the *Wolbachia* strains are compatible with both doubly infected males and singly infected males of either strain. Doubly infected males are only compatible with doubly infected females (Sinkins et al., 2005). Here CI occurs if sperm from doubly infected males fertilize an egg infected with one of the *Wolbachia* strains (Poinsot et al., 2003; reviewed in Harris and Braig, 2003). To date there have been no rescue or modification factors identified.

There are many events during spermatogenesis in which *Wolbachia* may potentially interact with the developing sperm cells. One possibility is that *Wolbachia* modifies translational control during the late stages of spermatogenesis, where expression is limited to a handful of genes necessary for sperm formation (reviewed in Harris and Braig, 2003). One aspect of spermatogenesis that *Wolbachia* may influence is the condensation of the sperm nucleus. Possibly, *Wolbachia* alters the interaction between protamine-like proteins and DNA, or modifies sperm nuclear basic proteins (SNBPs) (Harris and Braig, 2003). Removal of SNBPs from the male pronucleus after fertilization may influence egg activation or initiation of DNA replication and transcription (Harris and Braig, 2003). The possibility that mature *Drosophila* sperm carry *Wolbachia* encoded

proteins into the cytoplasm of the egg during fertilization has not been demonstrated (Sasaki et al., 1998; Kamoda et al., 2000).

A number of potential molecular mechanisms, including malfunction of nuclear envelope breakdown, mitotic chromosome condensation, replacement of SNBPs with maternally supplied histones, or the regulation of cell-cycle proteins may induce CI post fertilization (Tram et al., 2003). Alternatively *Wolbachia* might induce CI through methylation of host sperm DNA which can delay chromatin remodeling post-fertilization. *Wolbachia* cells are directly in contact with the membranes of spermatid tails in *Nasonia vitripennis* (Ashmead) (Hymenoptera: Pteromalidae). Bordenstein et al. (2006) suggest that this physical contact may have a modifying effect on the developing spermatids.

The male killing phenotype has been described in hosts belonging to different orders of arthropods including Coleoptera (Hurst et al., 1999, Fialho and Stevens, 2000), Lepidoptera (Hurst et al., 1999, Jiggins et al., 2001, Dyson et al., 2002), Diptera (Dyer and Jaenike, 2004, Hurst et al., 2000) and Pseudoscorpionida (Zeh et al., 2005). Male killing occurs when females infected with *Wolbachia* produce a female sex-ratio bias as a result of male death occurring at some stage of embryonic development. The male killing phenotype can be induced by a number of different bacteria in addition to *Wolbachia* including *Flavobacteria* (Hurst et al., 1999b), *Spiroplasma* (reviewed in Hurst et al., 2003), *Arsenophonus* (Werren et al., 1986) and *Rickettsia* (reviewed in Perlman et al., 2006), although it is not clear if the cellular mechanism is similar in all cases. In *Drosophila bifasciata* (Pominini) (Diptera, Drosophilidae), *Wolbachia* infection leads to defects of

chromosome condensation and segregation in male embryos only, resulting in death (Riparbelli et al., 2012). These defects are similar to those effects seen in *Wolbachia*-induced CI in *Drosophila*. *Wolbachia* strains transferred between different *Drosophila* species result in a transition between CI and male killing phenotypes, suggesting a shared mechanism for inducing each phenotype (Jaenike, 2007). The exact molecular mechanism for *Wolbachia*-induced alteration of chromatin organization specifically in male embryos has not been identified.

Parthenogenesis inducing strains of *Wolbachia*, found mainly in Hymenoptera, are associated in insects with a haplodiploid sex determination system. In Hymenoptera this sex determination results in mainly arrhenotoky, in which unfertilized eggs develop into haploid males, and fertilized eggs develop into diploid females. Less common is thelytoky in which no males occur in a population and all eggs develop into females (reviewed in Heimpel and de Boer, 2008). In Hymenoptera, *Wolbachia* infection induces thelytokous parthenogenesis in populations where chromosome duplication leads to diploidization of the haploid egg resulting in all female broods. This *Wolbachia*-induced diploidy restoration has been shown to occur by two processes, either by skipping the first mitotic anaphase seen in *Trichogramma* sp. (Hymenoptera, Trichogrammatidae), and *Leptopilina clavipes* (Hartig) (Hymenoptera: Figitidae) (Pannebakker et al., 2004; Stouthamer and Kazmer, 1994) or by fusion of daughter nuclei after the first mitotic division as seen in *Diplolepis rosae* (L.) (Hymenoptera: Cynipidae) and *Muscidifurax uniraptor* (Kogan and Legner) (Hymenoptera: Pteromalidae) (Stille and Davring, 1980; Gottlieb et al., 2002).

Another reproductive phenotype induced by *Wolbachia* is feminization, where genetic males are converted to functional females resulting in all female progeny. Feminization was originally identified in isopods. In these crustaceans, *Wolbachia* prevents the development of the androgenic gland by an unknown mechanism, thereby preventing male sex determination during development (Reviewed in Cordaux et al., 2011).

There is no phylogenetic relationship between *Wolbachia* strains and the various types of induced reproductive phenotypes. When a *Wolbachia* strain is transferred to a novel host species, it may cause a different phenotype. For example, when the feminizing *Wolbachia* strain infecting the Asian corn borer *Ostrinia furnacalis* (Guenée) (Lepidoptera: Crambidae) is transfected to *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae), it causes male killing in the latter (Fujii et al., 2001). One strain of *Wolbachia* that causes CI in *Cadra cautella* (Walker) (Lepidoptera: Pyralidae), induces male killing in *E. kuehniella* (Sasaki et al., 2002). This suggests that the reproductive phenotype caused by *Wolbachia* is determined in part by the molecular interaction between the host and bacteria.

Fitness effects of *Wolbachia*

The effects of a *Wolbachia* infection are not limited to altering host reproduction; both positive and negative fitness effects have been identified in some but not all insect systems. For example, *Wolbachia* infection increases fitness in the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Xue et al., 2012), the mosquito, *Aedes albopictus* (Skuse) (Diptera: Culicidae)

(Dobson et al., 2002), and the fly, *D. simulans* (Weeks et al., 2007). In *B. tabaci*, the presence of *Wolbachia* results in a reduced development time and increased survival rate of nymphs and an overall increased lifespan (Xue et al., 2012). In *A. albopictus* both singly and doubly infected females have increases in fecundity, egg hatch rate, and lifespan (Dobson et al., 2002).

With *D. simulans* an increase in fecundity has been shown (Weeks et al., 2007) however this has resulted in *Wolbachia* shifting from parasitism to mutualism in California populations. The presence of *Wolbachia* inducing CI in natural *D. simulans* populations was first identified by Hoffmann et al. (1986) as confined to southern California. *Wolbachia* was able to spread northward through populations as a result of CI (Turelli and Hoffmann, 1991; Turelli and Hoffmann, 1995). Initially, the relative cost of this *Wolbachia* infection was a 10-20% reduction in fecundity in the laboratory (Hoffmann et al., 1990). Currently a *Wolbachia* infection in *D. simulans* results in a fecundity advantage, shifting from the previously described fitness cost (Weeks et al., 2007).

Wolbachia confers resistance to parasites and pathogens. In *B. tabaci, Wolbachia* infection protects against the parasitoid *Encarsia bimaculata* (Heraty and Polaszek) (Hymenoptera: Aphelinidae) where a decrease in the success of parasitoid development was seen in infected hosts (Xue et al., 2012). In the parasitoid *Microtonus aethiopoides* (Loan) (Hymenoptera: Braconidae), *Wolbachia* infection results in protection against the immune defense of the host, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) (Hsaio 1996). In contrast, an increase in susceptibility has been observed in *Wolbachia*-infected *D. simulans*

to the parasitoid *Leptopilina heterotoma* (Thomson) (Hymenoptera: Eucoilidae) (Fytrou et al., 2006).

Resistance to RNA viruses is conferred by a *Wolbachia* infection in *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) (Teixeira et al., 2008), *Culex quinquefasciatus* (Say) (Diptera:Culicidae) (Glaser and Meola 2010, Bian et al., 2010) and in *Aedes aegypti* (L.) (Diptera: Culicidae) (Hussain et al., 2013). The combined parasite and virus resistance due to a *Wolbachia* infection is seen in *A. aegypti.* Resistance to the RNA viruses causing Dengue and Chikungunya, and the protozoan parasite *Plasmodium gallinaceum* was described by Moreira et al. (2009). *Wolbachia* has also been shown to confer resistance to *Plasmodium falciparum* in *Anopheles stephensi* (Liston) (Diptera: Culicidae) (Bian et al., 2013).

Wolbachia in filarial nematodes acts as an obligate mutualist. *Wolbachia* are essential for embryogenesis as well as worm development and survival (Pfarr and Hoerauf, 2007). Comparison of the genome of *Wolbachia* strain *w*Bm with its host *Brugia malayi* (Brug) (Spirurida: Onchocercidae) revealed that the metabolic pathways for nucleotide, heme, riboflavin and flavin adenine dinucleotide (FAD) biosynthesis are present in *Wolbachia* but are not found in the host (Foster et al., 2005). Similarily the *Wolbachia w*Oo in the host *Onchocerca ochengi* (Spirurida: Onchocercidae) retains the pathways for nucleotide and heme biosynthesis but lacks enzmyes in the riboflavin pathway (Darby et al., 2012).

In the bedbug *Cimex lectularius* (L.) (Hemiptera: Cimicidae), *Wolbachia* is localized in specialized organs called bacteriomes and acts as an obligate

mutualist. Antibiotic elimination of *Wolbachia* from the bedbug hosts results in a reduction of egg development and adult emergence with an increase in nymphal development time. This effect was reversed when blood meals were supplemented with B-vitamins (Hosokawa et al., 2010).

In the parasitic wasp, *Asobara tabida* (Nees) (Hymenoptera, Braconidae), the presence of *Wolbachia* is essential to normal oocyte production in females, but has no observable effect on male fertility. The relationship in females is related to *Wolbachia* density. Increasing the concentration of antibiotics resulted in reduced *Wolbachia* numbers and decreasing oocyte load. In this insect *Wolbachia* prevents apoptosis of nurse cells (Dedeine et al., 2001).

In some insects, indirect fitness effect of a *Wolbachia* infection can be detected. In infected *D. melanogaster* reared on normal cornmeal diets, the loss or addition of iron from the diet results in increased fecundity in *Wolbachia*-infected flies (Brownlie et al., 2009). The heme biosynthetic pathway is conserved in *Wolbachia* strains sequenced to date. This provides evidence of the potential role of *Wolbachia* in maintaining iron homeostasis.

Wolbachia **phylogeny**

The phylogenetic placement of *Wolbachia* in the order Rickettsiales is based on 16S rRNA sequence data (Figure 1.1 adapted from Williams et al., 2007). *Wolbachia* is closely related to other obligate intracellular bacteria such as *Neorickettsia risticii* (formerly *Ehrlichia risticii*), *Anaplasma marginale* and *Rickettsia spp*. These intracellular bacteria are known mammalian pathogens that

are vectored by insects. It is estimated based on *ftsZ* (cell division protein) sequence divergence that the common ancestor of *Wolbachia* from *Ehrlichia* occurred 142-162 million years ago (Werren et al., 1995; Anderson and Karr, 2001). Currently ten supergroups comprise the genus *Wolbachia* (Ros et al., 2009). Groups A, B, and F include *Wolbachia* from arthropods; groups C, D, and J are from filarial nematodes (Bandi et al., 1998; Ros et al., 2009); E and H supergroups are from springtails and termites respectively; supergroup K is found only in the mite suborder Prostigmata; supergroup I is found within the order Siphonaptera (fleas). Supergroup G was initially described for strains infecting spiders in the genus *Diaea*, but this is currently disputed as 16S data from these individuals is the result of recombination between strains from supergroups A and B (Baldo and Werren, 2007; Ros et al., 2009)

Phylogenetic analysis of the *Wolbachia* strains from supergroup C and D shows congruence with their host phylogeny, indicating coevolution between these strains and their host (Bandi et al., 1998). Nematodes belonging to the family Onchocercidae are the only hosts of *Wolbachia* groups C and D.

Phylogenetic data for *Wolbachia* strains infecting arthropods show a lack of congruence between symbiont and host. In insects, there is no obvious relationship between the *Wolbachia* phylogeny and induced reproductive phenotypes. Although the most common route of transmission for *Wolbachia* in insects is vertical and transovarial from mother to offspring, strict vertical transmission leads to congruent phylogenies. In contrast phylogenetic evidence shows that horizontal transmission of *Wolbachia* between insect host species

occurs over an evolutionary timeframe (Pfarr and Hoerauf, 2007; Vavre et al., 1999, Werren et al., 1995). However the lack of congruence may also be the result of other biological processes, such as recombination, as seen in supergroup G (Paptsova and Gogarten, 2007). Unlike some supergroups of *Wolbachia* which only infect a single taxon, *Wolbachia* strains belonging to groups A and B have a diverse host range and different arthropod hosts can carry the same strain. In addition a single host may carry a *Wolbachia* strain from both A and B supergroups simultaneously (Tram et al., 2003).

Phylogenetic relationships between *Wolbachia* strains were thought to be resolved with the sequencing of the DNA coding for *Wolbachia* surface protein (*wsp*) (Zhou et al., 1998), due to its high sequence variability. This became the *de facto* standard for *Wolbachia* phylogenetic studies. However there are discrepancies between *Wolbachia* phylogenies generated using *wsp* compared to those generated using *ftsZ* sequences. These discrepancies result from extensive recombination of hypervariable regions within the *wsp* gene which is therefore unsuitable for strain typing (Jiggins et al., 2001, Baldo et al., 2005). A multilocus approach based on five conserved genes (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*) has been applied in order to reliably type different *Wolbachia* strains (Baldo et al., 2006).

Wolbachia infection in agricultural pests

Wolbachia-induced reproductive phenotypes are potentially useful for biological control. One strategy uses CI as a method to suppress populations,

using mass release of incompatible males into wild populations. This method, similar to the sterile insect technique (SIT), is able to suppress populations; examples of this method have been employed in *C. pipiens* (Laven, 1967), *Aedes polynesiensis* (Marks) (Diptera: Culicidae) (O'Connor et al., 2012), and the medfly *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Zabalou et al., 2004). The benefit of using *Wolbachia* to surppress populations is that naturally infected males do not have reduced fitness resulting from irradiation or chemicals to sterilize males used in traditional SIT approaches (Alam et al., 2011). *Wolbachia*-induced parthenogenesis strains in hymenopteran parasitoids can reduce pest populations. The ability of *Wolbachia* to generate all-female populations allows for easier mass rearing of parasitoids, and colonization in new areas (Stouthamer, 1993). This thesis examines *Wolbachia* infecting agriculturally important pests within the family Curculionidae and family Plutellidae as well as their natural parasitoid enemies.

Weevils are phytophagous insects belonging to the family Curculionidae, and are associated with virtually all freshwater or terrestrial plant taxa (Anderson, 1993). *Wolbachia* has been identified in numerous species of Curculionidae, mainly in the agricultural pests. Examination of 40 weevil species in central Europe found that 40% of species were infected with *Wolbachia*, with a higher frequency of infection in parthogenetic weevils (Lachowska et al., 2010). A survey of broad-nosed weevils (Entiminae) found a 72% infection rate among 29 different species and identified a correlation between *Wolbachia* infection and parthenogenetic reproduction (Rodriguero et al., 2010). Both of these studies

showed that weevils could be infected by strains from either the A or B supergroup or coinfected by both. The phylogenetic data showed that horizontal transfer of *Wolbachia* has occurred in these systems (Lachowska et al., 2010, Rodriguero et al., 2010). In addition to being infected with *Wolbachia*, the presence of primary endosymbionts in the the rice weevil *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) is an example of coordination of multiple genomes directing the biology of a single host (Heddi et al., 1999).

There are numerous examples of *Wolbachia* being required by members of Curculionidae for successful egg development. *Wolbachia* has been implicated in oogenesis in the rice water weevil, *Lissorhoptrus oryzophilus* (Kuschel) (Coleoptera: Curculionidae) where number of eggs laid and hatch rate are negatively affected in antibiotic-treated females (Chen et al., 2012). In the date stone beetle, *Coccotrypes dactyliperda* (Fabricius) (Coleoptera: Curculionidae) mature oocytes were not produced by antibiotic-treated females (Zchori-Fein et al., 2006). In the black vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae), *Wolbachia* is also required for normal egg development (Son et al., 2008). The involvement of *Wolbachia* in egg development was initially described in the wasp *A. tabida* (Dedeine et al., 2001). Whether these different *Wolbachia* strains have a shared mechanism to modify oogenesis has not been identified.

Wolbachia infects members of various families of Lepidoptera, including important agricultural pests. Surveys examining the prevelance of *Wolbachia* in populations of Lepidoptera found 45% infection rate among 49 species in Japan

(Tagami and Miura, 2004), 52% infection rate among 56 species from five families of Lepidoptera in India (Salunke et al., 2012); 56% infection rate from 16 species of mulberry pests in 4 families (Pattabhiramaiah et al., 2012). These studies showed that lepidopteran hosts can be infected by strains from either the A or B supergroup.

Genome characteristics

Entire genomes of five *Wolbachia* strains have been sequenced including *Wolbachia* from *D. melanogaster* (*w*Mel, 1.27 Mb) (Wu et al., 2004), *B. malayi* (*w*Bm, 1.08Mb) (Foster et al., 2005), *C. quinquefasciatus* Pel (*w*Pip, 1.48Mb), (Klasson et al., 2008), *C. quinquefasciatus* JHB (wPip,1.54Mb) (Salzberg et al., 2009), *D. simulans* (*w*Ri, 1.3Mb) (Klasson et al., 2009) and *O. ochengi* (*w*Oo, 0.96Mb) (Darby et al., 2012) with several more genome sequencing projects currently underway for *Wolbachia* infecting *Drosophila willistoni*, *D. ananassae*, *N. vitripennis*, *M. uniraptor*, and *C. pipiens* molestus (Benson et al., 2013) .

These sequencing studies show shared characteristics between genomes. One characteristic similar to other obligate endosymbionts is a reduced genome size compared to genomes of related free-living bacteria (e.g. *Escherichia coli*, 4.6Mb). The reduced genome size is a consequence of *Wolbachia*'s obligate intracellular lifestyle, and results from the accumulation of deletions and the loss of nonessential DNA through genomic rearrangement.

Despite these reductions the genome of *w*Mel contains a large amount of repetitive DNA and a large number of mobile genetic elements including

bacteriophages (Wu et al., 2004). Some of these mobile genetic elements correspond to insertion sequences and transposons which may change or disrupt the function of genes and may contribute to the variety of phenotypically distinct *Wolbachia* strains (Wu et al., 2004). The presence of mobile genetic elements is a trait that is common to the genomes of only arthropod strains of *Wolbachia*. The numerous transposable elements, insertion sequences and prophage elements account for much of the genetic diversity between *Wolbachia* genomes. *Wolbachia* genomes also show evidence of extensive recombination. Most recombination events occur within supergroups rather than between them (Ellegaard et al., 2013). The presence of the genes required for homologous recombination has been indentified in the *Wolbachia* genome wMel (Wu et al., 2004) and the highest level of recombination observed in intracellular bacterial genomes has been documented within supergroup A *Wolbachia* (Klasson, 2009). The reduced amount of recombination between supergroups is likely the result of the accumulation of genome rearrangement and insertions. *Wolbachia* genomes within a supergroup have larger regions of collinearity than between supergroups, suggesting that this genomic divergence contributes to reduced frequency of recombination between supergroups (Ellegaard et al., 2013).

Endosymbiotic phages – role in symbiont biology

Prophage sequences are absent from strictly mutualistic endosymbionts such as *Wigglesworthia glossinidia* (Ackman et al., 2002), *Blochmannia pennsylvanicus* (Degnan et al., 2005), *Carsonella ruddii* (Nakabachi et al., 2006)

and *Buchnera aphidicola* (van Ham et al., 2003). In contrast, numerous prophages have been found in the genomes of facultative endosymbiotic bacteria. Intact phages and phage remnants in insect endosymbionts include the APSE phage of *Hamiltonella defense* (van der Wilk et al., 1999), APSE-related phages of *Arsenophonus* spp. (Hansen et al., 2007), prophage islands in *Regiella insecticola* (Degnan et al., 2010), SG1 (pSOG3) of *Sodalis glossinidius* (Clark et al., 2007), spV3 of *Spiroplasma spp* (Cohen et al., 1987), and phage genes in *Serratia symbiotica* (Burke and Moran, 2011).

In some cases, these phages have a beneficial effect for the host insect, for example, by conferring parasitoid resistance. The ability of a *H. defensa* infection to protect the aphid *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) from parasitoids is not dependent on bacterial chromosomal encoded proteins but on proteins found within the genome of the phage APSE (Degnan and Moran, 2008). Specifically, it is the presence of a virulence cassette in the ASPE genome, encoding toxins that allows for immunity to parasitization (Degnan and Moran, 2008). One such toxin is a Cytolethal Distending Toxin b-subunit (CDT), described in numerous mammalian bacterial pathogens which has nuclease activity and leads to chromatin fragmentation and cell cycle arrest of the eukaryotic nucleus (Lara-Tejero and Galán, 2000). Other APSE toxins are homologous to a shiga toxin which is involved in protein biosynthesis inhibition in mammals (Johannes and Römer, 2010). The loss of APSE phage in *H. defensa* in laboratory-reared colonies of aphids resulted in loss of parasitoid resistance (Oliver et al., 2009). In contrast in *R. insecticola,* where ASPE is not found,

bacterial encoded pathogenicity factors are responsible for protecting *A. pisum* from parasitoids (Hansen et al., 2012). Toxins in this group include repeats-in-toxin (RTX) proteins and two secretion systems. Although it is unknown how these pathogenicity factors specifically target and prevent parasitoid development, absence of these factors results in loss of protection (Hansen et al., 2012)..

Wolbachia genomes contain numerous prophage regions, prophage remnants and pyocin like elements. The prophage WO was first identified by Masui et al. (2000) in *Wolbachia* infecting *Teleogryllus taiwanemma* (Ohmachi and Matsumura) (Orthoptera: Gryllidae) and has since been found in almost every sequenced *Wolbachia* genome with the exception of the *Wolbachia* endosymbionts infecting the filarial parasites. Toxin proteins found in many sequenced WO genomes include patatin-like phospholipase and YD-peptide repeat protein, however how these proteins are involved in maintaining or establishing symbiosis of *Wolbachia* or its associated reproductive phenotypes have not been examined (Kent and Bordenstein, 2010)

Objectives

The acquisition of endosymbiotic bacteria by a host presents an opportunity for the host to utilize a novel genome sequence that may be beneficial to its own biology, as seen in mutualistic bacteria of arthropods, such as *Buchnera* in aphids. In contrast, cost and benefits of a *Wolbachia* infection to host insects are not always obvious. *Wolbachia* symbiosis provides a system where horizontal transfer of intracellular bacteria between unrelated hosts, horizontal transfer of

bacteriophages between bacteria and the horizontal gene transfer among three organisms can be studied.

Currently, knowledge of horizontal transfer of genes and genomes between bacterial endosymbionts and their bacteriophage and insect hosts is limited. The broad purpose of this study is to examine the mobility of *Wolbachia* and its WO prophage using phylogenetic and molecular tools.

There are three specific objectives in this thesis:

(i) Identify the movement and prevalence of specific *Wolbachia* strains and WO prophages between invasive agricultural pests and their parasitoids. This multipartite system involves the genomes of host insect, parasitoids, bacterial symbionts and integrated prophages.

(ii) Identify the core genome of replicating bacteriophage WO in recently sequenced *Wolbachia* genomes. Replicating WO has been implicated in the high rate of recombination in *Wolbachia.*

(iii) Examine one unique WO prophage gene, an adenine methyltransferase by determining its phylogenetic relationships. Examine the extent of horizontal transfer of this gene between bacterial hosts. Analyze the genomic neighborhood to determine the putative role of this gene in *Wolbachia*.

Figures

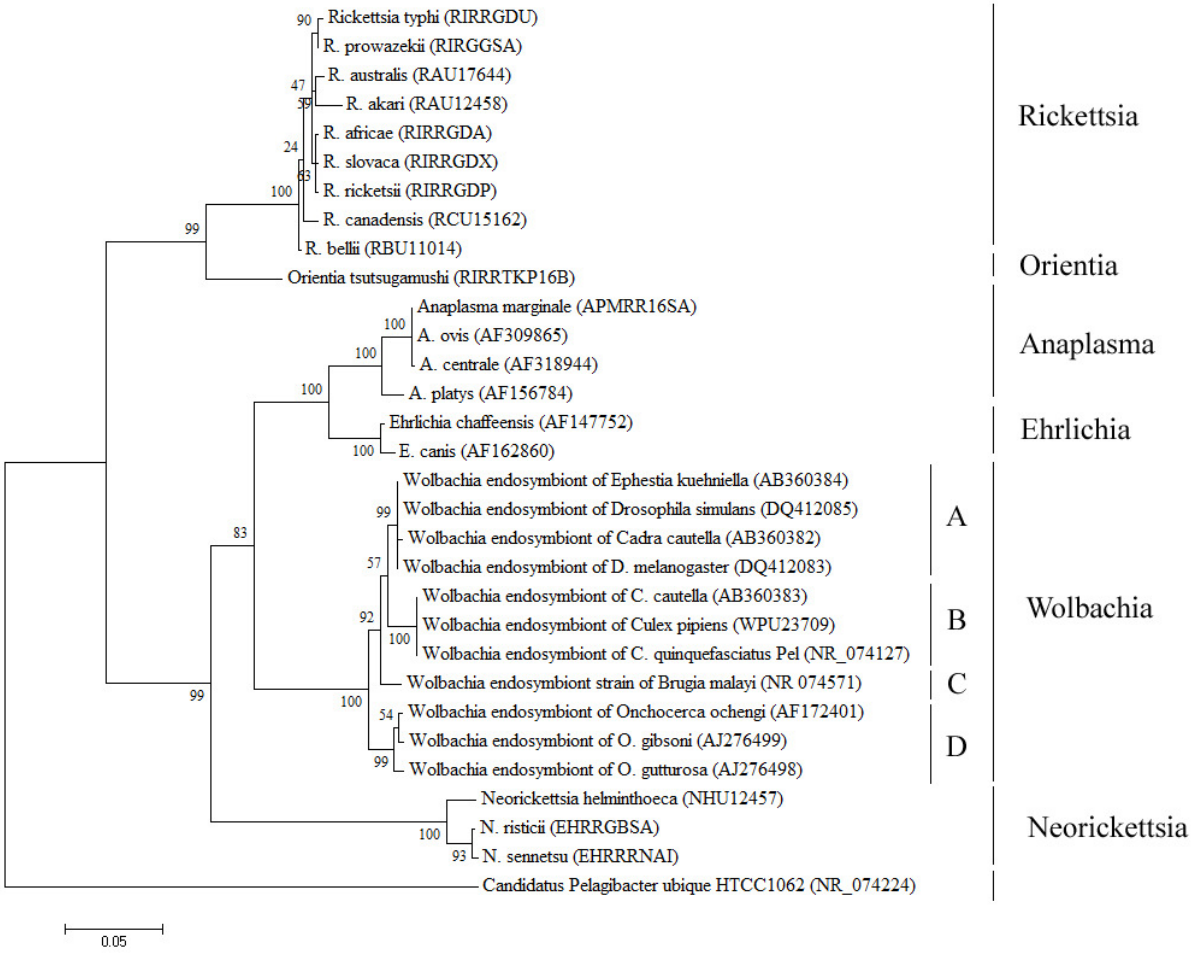

Rickettsia typhi (RIRRGDU)
R. prowazekii (RIRGGSA)
R. australis (RAU17644)
R. akari (RAU12458)
R. africae (RIRRGDA)
R. slovaca (RIRRGDX)
R. ricketsii (RIRRGDP)
R. canadensis (RCU15162)
R. bellii (RBU11014)
Orientia tsutsugamushi (RIRRTKP16B)
Anaplasma marginale (APMRR16SA)
A. ovis (AF309865)
A. centrale (AF318944)
A. platys (AF156784)
Ehrlichia chaffeensis (AF147752)
E. canis (AF162860)
Wolbachia endosymbiont of Ephestia kuehniella (AB360384)
Wolbachia endosymbiont of Drosophila simulans (DQ412085)
Wolbachia endosymbiont of Cadra cautella (AB360382)
Wolbachia endosymbiont of D. melanogaster (DQ412083)
Wolbachia endosymbiont of C. cautella (AB360383)
Wolbachia endosymbiont of Culex pipiens (WPU23709)
Wolbachia endosymbiont of C. quinquefasciatus Pel (NR_074127)
Wolbachia endosymbiont strain of Brugia malayi (NR 074571)
Wolbachia endosymbiont of Onchocerca ochengi (AF172401)
Wolbachia endosymbiont of O. gibsoni (AJ276499)
Wolbachia endosymbiont of O. gutturosa (AJ276498)
Neorickettsia helminthoeca (NHU12457)
N. risticii (EHRRGBSA)
N. sennetsu (EHRRRNAI)
Candidatus Pelagibacter ubique HTCC1062 (NR_074224)
Rickettsia
Orientia
Anaplasma
Ehrlichia
A
B
C
D
Wolbachia
Neorickettsia
0.05

Figure 1.1. Phylogeny of *Wolbachia* within the order Rickettsiales adapted from Williams et al. (2007). Evolutionary analyses were conducted in MEGA5 using the maximum likelihood method based on the General Time Reversible model. All positions containing gaps and missing data were eliminated. Bootstrap values from 100 replicates are shown next to the branches.

References

Akman L, Yamashita A, Watanabe H, Oshima K, Shiba T, Hattori M, Aksoy S. 2002. Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. Nature Genetics, 32(3):402-7.

Alam U, Medlock J, Brelsfoard C, Pais R, Lohs C, Balmand S, Carnogursky J, Heddi A, Takac P, Galvani A, Aksoy S. 2011. *Wolbachia* symbiont infections induce strong cytoplasmic incompatibility in the tsetse fly *Glossina morsitans*. PLoS Pathogens 7(12): e1002415.

Anderson, R. S., 1993: Weevils and plants: phylogenetic versus ecological mediation of evolution of host plant associations in Curculioninae (Coleoptera: Curculionidae). Memoirs of the Entomological Society of Canada 125(165): 197-232

Anderson CL, Karr TL. 2001. *Wolbachia*: evolutionary novelty in a rickettsial bacteria. BMC Evolutionary Biology, 1:10.

Baldo L, Werren JH. 2007. Revisiting *Wolbachia* supergroup typing based on WSP: spurious lineages and discordance with MLST. Current Microbiology, 55(1):81-7.

Baldo, L., Lo, N., Werren, J.H. 2005. Mosaic nature of the *Wolbachia* surface protein. Journal of Bacteriology, 187:5406-5418.

Baldo L, Dunning Hotopp JC, Jolley KA, Bordenstein SR, Biber SA, Choudhury RR, Hayashi C, Maiden MC, Tettelin H, Werren JH. 2006. Multilocus sequence typing system for the endosymbiont *Wolbachia* pipientis. Applied Environmental Microbiology, 72(11):7098-110.

Bandi C, Anderson TJ, Genchi C, Blaxter ML. 1998. Phylogeny of *Wolbachia* in filarial nematodes. Proceedings of the Royal Society of London. Series B, 265(1413):2407-13.

Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. 2013. GenBank. Nucleic Acids Research, 41(Database issue):D36-42.

Bian G, Xu Y, Lu P, Xie Y, Xi Z. 2010. The Endosymbiotic bacterium *Wolbachia* induces resistance to Dengue virus in *Aedes aegypti*. PLoS Pathogens 6(4): e1000833.

Bordenstein SR, Marshall ML, Fry AJ, Kim U, Wernegreen JJ. 2006. The tripartite associations between bacteriophage, *Wolbachia*, and arthropods. PLoS Pathogens;2(5):e43

Brownlie JC, Cass BN, Riegler M, Witsenburg JJ, Iturbe-Ormaetxe I, McGraw EA, O'Neill SL. 2009. Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. PLoS Pathogens, 5(4): e1000368.

Burke GR, Moran NA. 2011. Massive genomic decay in *Serratia symbiotica*, a recently evolved symbiont of aphids. Genome Biology and Evolution, 3:195-208.

Chen SJ, Lu F, Cheng JA, Jiang MX, Way MO. 2012. Identification and biological role of the endosymbionts *Wolbachia* in rice water weevil (Coleoptera: Curculionidae). Environmental Entomology, 41(3):469-77.

Clark AJ, Pontes M, Jones T, Dale C. 2007. A possible heterodimeric prophage-like element in the genome of the insect endosymbiont *Sodalis glossinidius*. Journal of Bacteriology, 189(7):2949-51.

Cohen AJ, Williamson DL, Oishi K. 1987. SpV3 viruses of *Drosophila spiroplasmas*. Israel Journal of Medical Sciences, 23(5):429-33.

Cordaux R, Bouchon D, Grève P: 2011. The impact of endosymbionts on the evolution of host sex-determination mechanisms. Trends in Genetics, 27:332-341

Darby AC, Armstrong SD, Bah GS, Kaur G, Hughes MA, Kay SM, Koldkjær P, Rainbow L, Radford AD, Blaxter ML, Tanya VN, Trees AJ, Cordaux R, Wastling JM, Makepeace BL. 2012. Analysis of gene expression from the *Wolbachia* genome of a filarial nematode supports both metabolic and defensive roles within the symbiosis. Genome Research, 22(12):2467-77.

Dedeine F, Vavre F, Fleury F, Loppin B, Hochberg ME, Bouletreau M. 2001. Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. Proceedings of the National Academy of Sciences USA, 22;98(11):6247-52.

Degnan PH, Lazarus AB, Wernegreen JJ. 2005. Genome sequence of *Blochmannia pennsylvanicus* indicates parallel evolutionary trends among bacterial mutualists of insects.Genome Research, 15(8):1023-33.

Degnan PH, Moran NA. 2008. Diverse phage-encoded toxins in a protective insect endosymbiont. Applied and Environmental Microbiology, 74(21):6782-91.

Degnan PH, Leonardo TE, Cass BN, Hurwitz B, Stern D, Gibbs RA, Richards S, Moran NA. 2010. Dynamics of genome evolution in facultative symbionts of aphids. Environmental Microbiology, 12(8):2060-9.

Dobson SL, Marsland EJ, Rattanadechakul W. 2002. Mutualistic *Wolbachia* infection in *Aedes albopictus*: accelerating cytoplasmic drive. Genetics, 160(3):1087-94.

Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, O'Neill SL. 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochemistry and Molecular Biology, 29(2):153-60.

Dyer KA, Jaenike J. 2004. Evolutionarily stable infection by a male-killing endosymbiont in *Drosophila innubila*: molecular evidence from the host and parasite genomes.Genetics, 168(3):1443-55.

Dyson EA, Kamath MK, Hurst GD. 2002. *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera: Nymphalidae): evidence for horizontal transmission of a butterfly male killer. Heredity, 88(3):166-71.

Ellegaard KM, Klasson L, Näslund K, Bourtzis K, Andersson SGE. 2013. Comparative genomics of *Wolbachia* and the bacterial species concept. PLoS Genetics 9(4): e1003381.

Fialho RF, Stevens L. 2000. Male-killing *Wolbachia* in a flour beetle. Proceedings of the Royal Society of London. Series B, 267(1451):1469-73.

Foster J, Ganatra M, Kamal I, Ware J, Makarova K, Ivanova N, Bhattacharyya A, Kapatral V, Kumar S, Posfai J, Vincze T, Ingram J, Moran L, Lapidus A, Omelchenko M, Kyrpides N, Ghedin E, Wang S, Goltsman E, Joukov V,

Ostrovskaya O, Tsukerman K, Mazur M, Comb D, Koonin E, Slatko B. 2005. The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. PLoS Biology, 3(4):e121.

Fujii Y, Kageyama D, Hoshizaki S, Ishikawa H, and Sasaki T. 2001. Transfection of *Wolbachia* in Lepidoptera: the feminizer of the adzuki bean borer *Ostrinia scapulalis* causes male killing in the Mediterranean flour moth *Ephestia kuehniella*. Proceedings of the Royal Society of London. Series B, 268(1469): 855–859.

Fytrou A, Schofield PG, Kraaijeveld AR, Hubbard SF. 2006. *Wolbachia* infection suppresses both host defence and parasitoid counter-defence. Proceedings of the Royal Society of London. Series B, 273(1588):791-6.

Glaser RL, Meola MA. 2010. The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. PLoS One, 5(8):e11977.

Gosalbes MJ, Latorre A, Lamelas A, Moya A. 2010. Genomics of intracellular symbionts in insects. International Journal of Medical Microbiology, 300(5):271-8.

Gottlieb Y, Zchori-Fein E, Werren JH, Karr TL. 2002.Diploidy restoration in *Wolbachia*-infected *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). Journal of Invertebrate Pathology, 81(3):166-74.

Harris HL, Braig HR. 2003. Sperm chromatin remodelling and *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila*. Biochemistry and Cell Biology, 81(3):229-40.

Hansen AK, Vorburger C, Moran NA. 2012. Genomic basis of endosymbiont-conferred protection against an insect parasitoid. Genome Research, 22(1):106-14.

Hansen AK, Jeong G, Paine TD, Stouthamer R. 2007. Frequency of secondary symbiont infection in an invasive psyllid relates to parasitism pressure on a geographic scale in California. Applied and Environmental Microbiology, 73(23):7531-5.

Heddi A, Grenier AM, Khatchadourian C, Charles H, Nardon P. 1999. Four intracellular genomes direct weevil biology: nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*. Proceedings of the National Academy of Sciences USA,96(12):6814-9.

Heimpel GE, de Boer JG. 2008. Sex determination in the Hymenoptera. Annual Review of Entomology, 53:209-30.

Hertig, M., Wolbach, S. 1924. Studies on rickettsia-like microorganisms in insects. Journal of Medical Research 44: 329–374.

Hertig, M. 1936. The rickettsia *Wolbachia* pipientis (gen. et sp.n) and associated inclusions of the mosquito, *Culex pipiens*. Parasitology 28: 453–486.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren, JH. 2008. How many species are infected with *Wolbachia*? – a statistical analysis of current data. FEMS Microbiology Letters, 281:215-220

Hsaio TH. 1996. Studies of interactions between alfalfa weevil strains, *Wolbachia* endosymbionts and parasitoids. In The Ecology of Agricultural Pests: Biochemical Approaches. Edited by W.O.C. Symondson and J.E. Liddell. Chapman and Hall, London, UK. pp. 51-71.

Hoffmann AA, Turelli M, Simmons GM. 1986. Unidirectional incompatibility between populations of *Drosophila simulans*. Evolution 40: 692–701.

Hoffmann AA, Turelli M, Harshman LG. 1990. Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. Genetics 126: 933–948

Hosokawa T, Koga R, Kikuchi Y, Meng XY, Fukatsu T. 2010. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. Proceedings of the National Academy of Sciences USA, 107(2):769-74.

Hurst GD, Jiggins FM, Majerus MEN. 2003. Inherited microorganisms that selectively kill male hosts: The hidden players of insect evolution?, In Insect Symbiosis. Edited by K. Bourtzis and T. A. Miller. CRC Press, Boca Raton, FL. pp. 177–198

Hurst GD, Bandi C, Sacchi L, Cochrane AG, Bertrand D, Karaca I, Majerus ME. 1999b. *Adonia variegata* (Coleoptera: Coccinellidae) bears maternally inherited flavobacteria that kill males only. Parasitology, 118 (Pt 2):125-34.

Hurst GD, Johnson AP, Schulenburg JH, Fuyama Y. 2000. Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. Genetics, 156:699–709.

Hurst GD, Jiggins FM, Schulenburg J H G von der, Bertrand D, West SA, Goriacheva II, Zakharov IA, Werren JH, Stouthamer R, Majerus MEN. 1999a . Male-killing *Wolbachia* in two species of insect. Proceedings of the Royal Society of London. Series B, 266(1420):735–735.

Hussain M, Lu G, Torres S, Edmonds JH, Kay BH, Khromykh AA, Asgari S. 2013. Effect of *Wolbachia* on replication of West Nile virus in a mosquito cell line and adult mosquitoes. Journal of Virology, 87(2):851-8.

Jaenike J. 2007. Spontaneous emergence of a new *Wolbachia* phenotype. Evolution, 61(9):2244-52.

Jiggins FM, Hurst GD, Majerus ME. 1998.Sex ratio distortion in *Acraea encedon* (Lepidoptera: Nymphalidae) is caused by a male-killing bacterium. Heredity, 81: 87–91

Jiggins FM, von Der Schulenburg JH, Hurst GD, Majerus ME. 2001. Recombination confounds interpretations of *Wolbachia* evolution. Proceedings of the Royal Society of London. Series B, 268(1474):1423-7.

Jiggins, F. M., Hurst, G. D., Schulenburg, J. H. & Majerus, M. E. 2001. Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*. Heredity, 86:161–166

Johannes L, Römer W. 2010. Shiga toxins--from cell biology to biomedical applications. Nature Reviews. Microbiology, 8(2):105-16.

Kamoda S, Masui S, Ishikawa H, Sasaki T. 2000. *Wolbachia* infection and cytoplasmic incompatibility in the cricket *Teleogryllus taiwanemma*. Journal of Experimental Biology, 203(Pt 16):2503-9.

Kent B, Bordenstein SR: Phage WO of *Wolbachia*: lambda of the endosymbiont world. Trends in Microbiology 2010, 18(4):173-181

Klasson L, Walker T, Sebaihia M, Sanders MJ, Quail MA, Lord A, Sanders S, Earl J, O'Neill SL, Thomson N, Sinkins SP, Parkhill J. 2008. Genome evolution of *Wolbachia* strain *w*Pip from the *Culex pipiens* group. Molecular Biology and Evolution, 25(9):1877-87.

Klasson L, Westberg J, Sapountzis P, Näslund K, Lutnaes Y, Darby AC, Veneti Z, Chen L, Braig HR, Garrett R, Bourtzis K, Andersson SG. 2009. The mosaic genome structure of the *Wolbachia* wRi strain infecting *Drosophila simulans*. Proceedings of the National Academy of Sciences USA, 106(14):5725-30

Lachowska, D., Kajtoch, L. and Knutelski, S. 2010. Occurrence of *Wolbachia* in central European weevils: correlations with host systematics, ecology, and biology. Entomologia Experimentalis et Applicata, 135: 105–118.

Landmann F, Orsi GA, Loppin B, Sullivan W. 2009. *Wolbachia*-mediated cytoplasmic incompatibility is associated with impaired histone deposition in the male pronucleus. PLoS Pathogens 5(3): e1000343.

Lara-Tejero M, Galán JE. 2000. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. Science, 290(5490):354-7.

Laven, H. 1967. Eradication of *Culex pipiens* fatigans through cytoplasmic incompatibility. Nature. 216: 383-384.

Masui S, Sasaki T, Ishikawa H. 2000. Genes for the type IV secretion system in an intracellular symbiont, *Wolbachia*, a causative agent of various sexual alterations in arthropods. Journal of Bacteriology, 182(22):6529-6531.

Masui, S., Kamoda, S., Sasaki, T., Ishikawa, H. 2000. Distribution and evolution of bacteriophage WO in *Wolbachia*, the endosymbiont causing sexual alterations in arthropods. Journal of Molecular Evolution, 51:491-497.

McGraw EA, O'Neill SL. 2004. *Wolbachia* pipientis: intracellular infection and pathogenesis in Drosophila. Current Opinion in Microbiology, 7(1):67-70.

Merçot H, Charlat S. 2004. *Wolbachia* infections in *Drosophila melanogaster* and *D. simulans*: polymorphism and levels of cytoplasmic incompatibility. Genetica, 120(1-3):51-9.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O'Neill SL. 2009. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. Cell, 139(7):1268-78.

Nakabachi A, Yamashita A, Toh H, Ishikawa H, Dunbar HE, Moran NA, Hattori M. 2006. The 160-kilobase genome of the bacterial endosymbiont *Carsonella*. Science, 314(5797):267.

O'Connor L, Plichart C, Sang AC, Brelsfoard CL, Bossin HC, Dobson SL. 2012. Open release of male mosquitoes infected with a *Wolbachia* biopesticide: field performance and infection containment. PLoS Neglected Tropical Diseases 6(11): e1797.

O'Neill, S. L. and T. L. Karr 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature 348, 78-80.

Oliver KM, Degnan PH, Hunter MS, Moran NA. 2009. Bacteriophages encode factors required for protection in a symbiotic mutualism. Science, 325(5943):992-4.

Pannebakker BA, Pijnacker LP, Zwaan BJ, Beukeboom LW. 2004. Cytology of *Wolbachia*-induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera: Figitidae). Genome, 47(2):299-303.

Pattabhiramaiah M, Reddy MS, Bruechkner D. 2012. Presence of *Wolbachia* in the Lepidopteran insect pests of mulberry. International Journal of Environmental Engineering Research; 1(1):16-22

Perlman SJ, Hunter MS, Zchori-Fein E. 2006. The emerging diversity of Rickettsia. Proceedings of the Royal Society B-Biological Sciences, 273: 2097–2106.

Poinsot D, Charlat S, Merçot H. 2003. On the mechanism of *Wolbachia*-induced cytoplasmic incompatibility: confronting the models with the facts. Bioessays, 25(3):259-65.

Poptsova MS, Gogarten JP. 2007. The power of phylogenetic approaches to detect horizontally transferred genes. BMC Evolutionary Biology, 21;7:45.

Pfarr KM, Hoerauf A. 2007. A niche for *Wolbachia*. Trends Parasitology, 23(1):5-7.

Rigaud T, Souty-Grosset C, Raimond R, Mocquard J, Juchault P. 1991. Feminizing endocytobiosis in the terrestrial crustacean *Armadillidium vulgare* Latr. (Isopoda): recent acquisitions.. Endocytobiosis and Cell Research, 7:259–73

Riparbelli MG, Giordano R, Ueyama M, Callaini G. 2012. *Wolbachia*-mediated male killing is associated with defective chromatin remodeling. PLoS ONE 7(1): e30045.

Rodriguero MS, Confalonieri VA, Guedes JV, Lanteri AA. 2010. *Wolbachia* infection in the tribe *Naupactini* (Coleoptera, Curculionidae): association between thelytokous parthenogenesis and infection status. Insect Molecular Biology, 19(5): 631-40.

Ros VI, Fleming VM, Feil EJ, Breeuwer JA. 2009. How diverse is the genus *Wolbachia*? Multiple-gene sequencing reveals a putatively new *Wolbachia* supergroup recovered from spider mites (Acari: Tetranychidae). Applied and Environmental Microbiology, 75(4):1036-43.

Salunke BK, Salunkhe RC, Dhotre DP, Walujkar SA, Khandagale AB, Chaudhari R, Chandode RK, Ghate HV, Patole MS, Werren JH, Shouche YS. 2012. Determination of *Wolbachia* diversity in butterflies from Western Ghats, India, by a multigene approach. Applied and Environmental Microbiology, 78(12):4458-67.

Salzberg SL, Puiu D, Sommer DD, Nene V, Lee NH. 2009. Genome sequence of the Wolbachia endosymbiont of Culex quinquefasciatus JHB. Journal of Bacteriology, 191(5):1725.

Sasaki T, Kubo T, Ishikawa H. 2002. Interspecific transfer of *Wolbachia* between two lepidopteran insects expressing cytoplasmic incompatibility: a *Wolbachia* variant naturally infecting *Cadra cautella* causes male killing in *Ephestia kuehniella*. Genetics, 162(3):1313-9.

Sinkins SP, Braig HR, O'Neill SL. 1995. *Wolbachia* superinfections and the expression of cytoplasmic incompatibility. Proceedings of the Royal Society of London. Series B, 261(1362):325-30.

Son, Y., Luckhart, S., Zhang, X., Lieber, M.J. and Lewis, E.E. 2008. Effects and implications of antibiotic treatment on *Wolbachia*-infected vine weevil (Coleoptera: Curculionidae). Agricultural and Forest Entomology. 10: 147–155.

Stille, B., and Davring, L. 1980. Meiosis and reproductive strategy in the parthenogenetic gall wasp *Diplolepis rosae* (L.) (Hymenoptera, Cynipidae). Hereditas, 92: 353-362.

Stouthamer R., J.A.J. Breeuwer, R. F. Luck, and J. H. Werren. 1993. Molecular identification of microorganisms associated with parthenogenesis. Nature 361:66-68.

Stouthamer, R. 1993. The use of sexual versus asexual wasps in biological control. Entomophaga 38: 3-6.

Stouthamer, R. and J. D. Kazmer. 1994. Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. Heredity 73:317-327.

Tagami Y, Miura K. 2004. Distribution and prevalence of *Wolbachia* in Japanese populations of Lepidoptera. Insect Molecular Biology, 13(4):359-64.

Teixeira, L., Ferreira, A., Ashburner, M. 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biology, 6: 2753-2763

Tram U, Ferree PM, Sullivan W. 2003. Identification of *Wolbachia*--host interacting factors through cytological analysis. Microbes and Infection, 5(11):999-1011.

Turelli M, Hoffmann AA. 1991. Rapid spread of an inherited incompatibility factor in California *Drosophila*. Nature 353: 440–442.

Turelli M, Hoffmann AA. 1995. Cytoplasmic incompatibility in *Drosophila simulans*: Dynamics and parameter estimates from natural populations. Genetics 140: 1319–1338.

Vavre, F., Fleury, F., Lepetit, D., Fouillet, P., Bouletreau, M. 1999. Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. Molecular Biology and Evolution, 16:1711-1723.

van der Wilk F, Dullemans AM, Verbeek M, van den Heuvel JF. 1999. Isolation and characterization of APSE-1, a bacteriophage infecting the secondary endosymbiont of *Acyrthosiphon pisum*. Virology, 262(1):104-13.

van Ham RC, Kamerbeek J, Palacios C, Rausell C, Abascal F, Bastolla U, Fernández JM, Jiménez L, Postigo M, Silva FJ, Tamames J, Viguera E, Latorre A, Valencia A, Morán F, Moya A. 2003. Reductive genome evolution in *Buchnera aphidicola*. Proceedings of the National Academy of Sciences USA, 100(2):581-6.

Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA. 2007. From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. PLoS Biology 5(5): e114.

Werren JH, Zhang W, Guo LR. 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proceedings of the Royal Society of London. Series B, 261(1360):55-63.

Werren JH, Skinner SW, Huger AM. 1986. Male-killing bacteria in a parasitic wasp. Science, 231(4741):990-2.

Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadinejad N, Wiegand C, Madupu R, Beanan MJ, Brinkac LM, Daugherty SC, Durkin AS, Kolonay JF, Nelson WC, Mohamoud Y, Lee P, Berry K, Young MB, Utterback T, Weidman J, Nierman WC, Paulsen IT, Nelson KE, Tettelin H, O'Neill SL, Eisen JA. 2004. Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biology, 2(3):E69.

Xue X, Li SJ, Ahmed MZ, De Barro PJ, Ren SX, Qiu BL. 2012. Inactivation of *Wolbachia* reveals Its biological roles in whitefly host. PLoS ONE 7(10): e48148.

Yen JH, Barr AR. 1973. The etiological agent of cytoplasmic incompatibility in *Culex pipiens*. Journal of Invertebrate Pathology, 22(2):242-50.

Yen JH, Barr AR. 1971. New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L. Nature, 232(5313):657-8.

Zabalou S, Riegler M, Theodorakopoulou M, Stauffer C, Savakis C, Bourtzis K. 2004. *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. Proceedings of the National Academy of Sciences USA, 101(42):15042-5.

Zchori-Fein, E., C. Borad, and A. Harari. 2006. Oogenesis in the date stone beetle, *Coccotrypes dactyliperda*, depends on symbiotic bacteria. Physiological Entomology. 31:164-169.

Zeh DW, Zeh JA, Bonilla MM. 2005. *Wolbachia*, sex ratio bias and apparent male killing in the harlequin beetle riding pseudoscorpion. Heredity, 95(1):41-9.

Zhou, W., Rousset, F., O'Neil, S. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. Proceedings of the Royal Society B: Biological Sciences, 265:509-515.

Chapter 2

Horizontal Transfer of *Wolbachia* in Host-Parasitoid Systems

2A: *Plutella xylostella* and *Diadegma insulare*

Introduction

Comparison of the phylogenies of *Wolbachia* with that of their hosts show little congruence, suggesting either that *Wolbachia* can be horizontally transferred between arthropod species (Werren et al., 1995; West et al., 1998) or that genetic recombination is occurring (Bordenstein and Wernegreen 2004; Baldo et al., 2005, 2006), or both. Possible routes for *Wolbachia* transfer include contact after injury (Rigaud and Juchault 1995) or through plants (Sintupachee et al., 2006) or parasitoids (Werren et al., 1995; Vavre et al., 1999).

Diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is an important agricultural pest of *Brassica* crops (Lamb 1989). The mustard family, Brassicaceae, comprises approximately 340 genera and over 3350 species, including important agricultural, ornamental, and research plants (Koch et al., 2003). The most economically important mustards are species of *Brassica* L., including various vegetable and oilseed crops (Koch et al., 2003). Numerous insect pests collectively can attack all above- and below-ground portions of *Brassica* crops. Above ground outbreaks of DBM can lead to almost complete crop loss due to defoliation while root maggots, *Delia radicum* (L.) (Diptera: Anthomyiidae), damage root systems and can lead to severe yield reductions

A version of this chapter has been published. Batista et al., 2010. *The Canadian entomologist.* 142: 57–64

(Soroka and Dosdall, 2011; Dosdall et al., 2012). DBM is well established throughout most of the United States, while infestations in Canada are reestablished annually by adult DBM transported by wind from the southern United States (Smith and Sears, 1982). As a result many growers rely on multiple insecticide applications to control infestations of DBM (Sarfraz et al., 2006). This intense use has resulted in resistance to virtually all known insecticides (Sarfraz and Keddie, 2005). Even with newer insecticides, such as spinosad and indoxacarb, a few years of extensive application can lead to resistance in certain DBM populations (Zhao et al., 2006). Because insecticides often adversely affect the natural enemies of DBM, these infestations are difficult to manage (Sarfraz et al., 2005). Pesticide resistance has made the use of parasitoids essential to sustainable management of DBM populations. Some species of the larval endoparasitoid genus *Diadegma* Förster (Hymenoptera: Ichneumonidae) have been used sporadically for biological control of DBM (Reviewed in Talekar and Shelton, 1993). One common species found in Canada is *D. insulare* (Cresson) (Sarfraz et al., 2005, 2006). This parasitoid apparently does not overwinter in temperate North America but likely disperses northward along with its hosts (Putnam, 1978; Dosdall et al., 2004a). *D. insulare* is the most successful biological control agent of DBM in Canada and is capable of parasitizing any larval instar of DBM (Dosdall et al., 2011). In Alberta, *D. insulare* accounts for the 45% of parasitism of DBM, with all other known DBM parasitoids accounting for <8% (Braun et al., 2004).

Recently, *Wolbachia* has been shown to provide resistance to RNA viruses, but not to DNA viruses, in *D. melanogaster* Meigen (Diptera: Drosophilidae) (Hedges et al., 2008; Teixeira et al., 2008); this may enhance the occurrence of this symbiont, already widespread in wild populations of *Drosophila* (Fallen). No evidence exists for this effect in DBM or *Diadegma*. However, if the same strain of *Wolbachia* is found in the host and the parasitoid as a result of horizontal transmission, and this strain provides resistance to RNA viruses, then DBM and *Diadegma* would gain the same benefit. The ability of *Wolbachia* to modify host immune response to parasitism has been shown previously in the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae), and *Drosophila simulans* (Sturtevant) (Diptera: Drosophilidae) (Hsiao, 1996, Ftrou et al., 2006). A *Wolbachia* infection in either the parasitoid or the host showed adverse immunity-related effects, resulting in reduced encapsulation of the parasitoid egg by the host, and reduced success of parasitization by the parasitoid (Fytrou et al., 2006). The success of the parasitoid development of *Microtonus aethiopoides* (Loan) (Hymenoptera: Braconidae), and *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae) is dependent on the absence of a *Wolbachia* infection in their host *Hypera postica* (Hsiao, 1996).

In this study I investigated (i) the phylogeny of *Wolbachia* found in Alberta populations of DBM and its parasitoid, *D. insulare*, using two *Wolbachia* housekeeping genes, groEl (heat-shock protein HSP60) and ftsZ (cell division protein), and (ii) the phylogeny of the WO bacteriophage, using the minor capsid protein gene orf7 (Gavotte et al., 2007). The selected housekeeping genes are

reliable for use in phylogenetic analysis because there is little or no detectable recombination within them (Ros et al., 2009). The objective was to determine if *Wolbachia* and the WO bacteriophage was horizontally transferred between host insect and parasitoid, phylogenetically distant species.

Materials and methods

Sample Collection

DBM and *D. insulare* were collected in commercial canola fields near Lethbridge in southern Alberta (49°38'N, 112°48'W). Laboratory colonies were established from field-collected insects. Following transport from the field sites adults were released into cages containing *Brassica napus* L. cv. Q2 (not shown). Each cage, (base 40.5 cm x 40.5 cm, height 80.5 cm, was lined on the sides and top with 500 um Nitex nylon at 20 ± 2 °C and kept under natural light (Figure 2.1). Cages were set up with potted *B. napus* plants where DBM adults oviposited. Parasitoids were released into a single cage when second instar larvae of the host were observed.

DNA extraction and sequencing

Genomic DNA was extracted from whole insects reared in the lab using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). The supergroup-A-specific primers *wsp*136f/*wsp*691r and supergroup-B-specific primers *wsp*81f/*wsp*522r (Braig et al., 1998, Zhou et al., 1998) were used initially to detect the presence of *Wolbachia* and determine its supergroup. Colonies

negative for *Wolbachia* were eliminated from further analysis; positive colonies were maintained for over a year before the phylogenetic analysis was conducted. The presence of the WO phage was tested with the *orf7* gene using the primers WOF and WOR (Masui et al., 2000). The *Wolbachia* ftsZ primers (Lo et al., 2002) and supergroup-B-specific groEL primers (Baldo et al., 2006) were used to amplify their respective regions (shown in Table 2.1). The polymerase chain reaction (PCR) products groEL, ftsZ, and orf7 were cloned into the pCR4-TOPO vector and inserted into One Shot TOP10-competent cells (TOPO TA Cloning Kit for Sequencing, Invitrogen). A consensus sequence was generated from three clones of every gene sequenced using a LI-COR 4300 DNA analyzer (LI-COR) with labeled primers and Thermo Sequenase labeled primer cycle sequencing kit (USB Corporation, Cleveland, Ohio) according to the manufacturer's instructions.

Phylogenetic Analyses

The *groEL* and *ftsZ* sequences were concatenated for each species prior to alignment (Gadagkar et al., 2005). These concatenated DNA sequences were added to a data set composed of *groEL* and *ftsZ* sequences previously used by Holden et al. (1993), Werren et al. (1995), Masui et al. (1997), Casiraghi et al. (2005), and Baldo et al. (2006). The *orf7* sequence genereated in this study were added to a data set of previously sequenced *orf7* genes by Masui et al. (2000), Bordenstein and Wernegreen (2004), and Gavotte et al. (2007). Both data sets were aligned with ClustalW and modified with BioEdit version 7.0.9 (Hall, 1999). The resulting alignment contained the largest overlap with *Wolbachia* from *D.*

insulare and *P. xylostella*. Phylogenetic trees were generated using PAUP* version 4.0b10 (Swofford, 2003) using the maximum parsimony and maximum likelihood methods. Maximum parsimony and maximum-likelihood heuristic searches were conducted with 100 random additions and tree bisection and reconnection branch swapping (TBR). Bootstrap values were calculated from 100 replicates using maximum-parsimony and maximum-likelihood method with 10 random taxon-addition replicates. Modeltest version 3.7 and Akaike's information criterion were used to select a DNA substitution model for each data set prior to maximum likelihood bootstrap analysis: *orf7* (TVM+G) and concatenated *ftsZ* and *groEL* (GTR+G) (Posada and Crandall, 1998). This was set in PAUP* for *ftsZ* and *groEL* data set (nucleotide frequencies A=0.34680, C=0.14410, G=0.23130, T=0.27780; gamma distribution of rates at variable sites; alpha shape parameter = 0.2582; rate categories = 4) and orf7 (nucleotide frequencies A=0.38240, C=0.12330, G=0.23110, T=0.26320; gamma distribution of rates at variable sites; alpha shape parameter = 0.6276; rate categories = 4). Due to the lack of a suitable outgroup both trees generated are midpoint-rooted.

For *wsp* sequence analysis, sequences were aligned with DBM *Wolbachia wsp* isolates found in previous studies (Delgado and Cook, 2009; Jeyaprakash and Hoy, 2000) Modeltest version 3.7 and Akaike's information criterion were used to select the GTR substitution model. Maximum likelihood analysis was conducted using PHYML with the GTR substitution model (estimated proportion of invariable sites, single substitution rate category). Bootstrap values were

calculated from 100 replicates replicates with NNI branch swapping (Guindon and Gascuel, 2003).

Results

PCR using *wsp*, *ftsZ*, *groEL*, and *orf7* primers showed that *D. insulare* and *P. xylostella* were each infected with the same *Wolbachia* strain and its WO phage. Phylogenetic analysis was performed to determine the evolutionary relationship of *Wolbachia* between DBM and *D. insulare*. Using maximum- parsimony and maximum-likelihood phylogenetic trees based on the concatenated alignment of from *Wolbachia* in DBM and *D. insulare*, a single *Wolbachia* strain belonging to supergoup B was detected (Figure 2.2).

The bacteriophage WO detected in this study is closely related to that found in *Wolbachia* in the parasitoid *Trichogramma kaykai* (Pinto and Stouthamer) (Hymenoptera:Trichogrammatidae) and its host *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) (Figure 2.3). My results show that the WO phage strains in DBM and *D. insulare* are 98% identical, with only three amino acid differences between the WO *orf7* translated product at positions 27 (Asp in DBM, Glu in *D. insulare*), 98 (Tyr in DBM, His in *D. insulare*), and 130 (Gly in DBM, Ser in *D. insulare*).

Phylogenetic analysis using DBM wsp sequences from Africa and Asia (Delgado and Cook, 2009) and United States (Jeyaprakash and Hoy, 2000) revealed a clustering of *Wolbachia* from DBM and *D. insulare* found in Alberta populations to the supergroup B family *Wolbachia* found in Malaysia and Kenya.

Discussion

This study, the only one to date to report on a Canadian DBM population, provides evidence that a single *Wolbachia* strain (from B-supergroup) was horizontally transferred between host and parasitoid. Previous phylogenetic studies have shown that there is often a lack of congruence between the phylogenies of *Wolbachia* and its hosts (Werren et al., 1995). Based on gene sequences, related strains of *Wolbachia* are found among phylogenetically distant insect hosts, which suggest horizontal transmission (Van Meer et al., 1999; Vavre et al., 1999; Werren et al., 1995). One mechanism for this transmission may be host-parasitoid associations. Van Meer et al. (1999), Vavre et al. (1999), and Gavotte et al. (2007) provide strong arguments for horizontal transfer, based on the phylogenetic relationships of *Wolbachia* strains in hymenopteran parasitoids with their hosts. Van Meer et al. (1999) found evidence for horizontal transmission between the egg parasitoids *T. kaykai* and *T. bourarachae* (Pintureau and Babault) and their host, *E. kuehniella*. Five instances of possible horizontal transmission were found by Vavre et al. (1999), including transfer between the larval parasitoids *Leptopilina heterotoma* (Thomson) (Hymenoptera: Eucoilidae) and *Asobara tabida* (Nees) (Hymenoptera: Braconidae) and their respective hosts, *D. simulans* and *D. melanogaster*. Vavre et al. (1999) also provided evidence for horizontal transfer between two different hymenopteran parasitoids, possibly through hyper- or multiparasitism, such as between two variants of *Trichopria* (Ashmead) (Diapriidae) and *L. heterotoma*, between the pupal parasitoid

Trichopria drosophilae (Perkins) (Hymenoptera: Diapriidae) and *A. tabida*, and between *Muscidifurax uniraptor* (Kogan and Legner) and *Pachycrepoideus vindemmiae* (Rondani) (Hymenoptera: Pteromalidae). Although phylogenetic studies have shown that the bacteriophage WO is frequently transferred horizontally between *Wolbachia* strains (Bordenstein and Wernegreen 2004, Gavotte et al., 2007), there are cases (e.g., between *T. kaykai* and *E. kuehniella* and between *M. uniraptor* and *Pachycrepoideus dubius* (Ashmed), where *Wolbachia* and its associated phage are transferred together (Gavotte et al., 2007). Consistent with above, the close phylogenetic relationship between *Wolbachia* and its phage in DBM and *D. insulare* indicates that they were transferred together between the two insect hosts (Figure 2.2, 2.3)

Wolbachia has previously been detected in DBM in the United States using the more sensitive long PCR technique (Jeyaprakash and Hoy, 2000). In this study, phylogenetic analysis based on wsp sequence data demonstrated that DBM had two strains of *Wolbachia*, *w*Sus-A1 and *w*Xyl-B1, belonging to the *w*Mel and *w*Con group in supergroups A and B, respectively (Jeyaprakash and Hoy, 2000). More recently, Delgado and Cook (2009) found three *Wolbachia* strains in DBM outside North America. Sequences from the dominant strain *plutWB1*, found in Africa and Asia, were about 17% divergent from sequences from the *w*Xyl-B1 strain. In my study, a single *Wolbachia* infection belonging to supergroup B was found in DBM and *D. insulare*. The wsp sequence data from my DBM population are the same as the *wsp* sequence data in DBM *plutWB1* (<1% sequence divergence) (Figure 2.4). Examining the phylogeny of host mtDNA haplotypes

Delgado and Cook (2009) showed two separate clades, individuals with *plutWB1* infection and the other clade with uninfected individuals. The presence of *plutWB1 Wolbachia* infection in two continents (now three with this study) and the monophyletic grouping of mtDNA haplotypes of infected individuals suggest a single ancestral infection of *Wolbachia* in DBM (Delgado and Cook, 2009). The difference in *wsp* sequence found in this DBM population and those previously described from United States (wXylB1) indicates the acquisition and replacement of *Wolbachia* strains in different populations of DBM in different geographic regions. The different *Wolbachia* strains harbored by these two populations of DBM in North America may indicate multiple colonization events by DBM into North America. Delgado and Cook (2009) also described sex-ratio distortion resulting from the presence of *plutWB1*. This aspect was not investigated in my study.

In addition to increasing the proportion of females in insect populations *Wolbachia* may influence host species in other ways. For example it inhibits RNA but not DNA virus infection *in D. melanogaster* (Hedges et al., 2008; Teixeira et al., 2008). Whether this leads to larger insect populations is unknown. *Wolbachia* has also been shown to modify the host immune response. In the alfalfa weevil, *H. postica,* rates of encapsulation of eggs of the larval parasitoid *B. curculionis* are higher when *Wolbachia* is present than when it is absent. In the same host, *Wolbachia* also influences the success rate of the adult parasitoid *M. aethiopoides*. Parasitoids develop normally in weevils without *Wolbachia* but are less successful in weevils with *Wolbachia* (Hsiao, 1996). As a consequence the

host weevil is protected from the adverse impacts of the parasitoid and the host population benefits. In a study where both host *D. simulans* and parasitoid *L. heterotoma* were infected with *Wolbachia*, adverse immunity related effects were detected in both insects (Fytrou et al., 2006). Encapsulation of parasitoid eggs in the infected host was reduced, but the presence of *Wolbachia* in the parasitoid alone reduced its success. The mechanisms of these effects have not been determined and it is uknown if they are virally mediated (Fytrou et al., 2006). This may be similar to the system I investigated. RNA virus replication in *Drosophila* spp. host is suppressed by *Wolbachia* (Teixeira et al., 2008); it is tempting to suggest that *Wolbachia* is interfering with viral replication in *L. heterotoma*. Reduction of the parasitoid virus (polydnavirus, a DNA virus) would make the injected eggs in an insect host more susceptible to encapsulation by hemocytes of the host immune system. The injected virus does not replicate in the host but generates RNA transcripts. The absence of viral transcriptional activity by polydnavirus would reduce or eliminate parasitoid development. In spite of the fact that the same strain of WO is present in *Wolbachia* in both DBM and *Diadegma*, further study is required to elucidate the role of the temperate dsDNA WO bacteriophage in host immune suppression or parasitization rate of DBM.

I have identified a single strain of *Wolbachia* and the bacteriophage WO in *P. xylostella* and *D. insulare*, which is strong evidence of horizontal transfer. Although the direction of horizontal transfer cannot be identified by phylogenetic analysis, the transmission of *Wolbachia* from insect host to parasitoid shown in previous studies where *Wolbachia* could be detected in the parasitoid *Leptopilina*

boulardi (Barbotin et al.) (Hymenoptera: Eucoilidae) after eclosing in *Wolbachia* infected *D. simulans* (Heath et al., 1999), suggests that this transfer has occurred from DBM to *D. insulare*. The complex relationship among viruses, bacteria, parasitoids, and hosts requires further study. Knowledge about this interaction may permit the development of effective pest-management strategies. Future investigation of *Wolbachia* dynamics may be an important factor in the control of DBM in the field and future use of *Wolbachia* as a vector for genetic transformation may depend upon WO lytic activity.

2B: *Ceutorhynchus obstrictus* and associated Parasitoids

Introduction

Cabbage Seedpod Weevil (CSW), *Ceutorhynchus obstrictus* (Marsham) (Coleoptera: Curculionidae), is an invasive species which is an important agricultural pest (Laffin et al., 2005). CSW was introduced from Europe almost 80 years ago. It has since spread into most of the continental United States, but more recently to Alberta in 1995 (Carcamo et al., 2001). The movement and source of this pest into North America was previously evaluated by sequencing the cytochrome oxidase I (COI) gene from mitochondrial DNA (mtDNA) from different source populations across Northern Europe and North America (Laffin et al., 2005). That survey of haplotypes identified two separate introductions of CSW; one into Quebec, which is the most recently established new population and remains genetically distinct from the rest of North American populations.

Alberta's CSW population consists of one haplotype. CSW has migrated from southern Alberta into southern Saskatchewan and Manitoba with the expectation that the entire canola growing region of western Canada will become infested (Dosdall et al., 2002; Dosdall et al., 2009).

In Alberta, overwintering CSW adults emerge in spring and feed on brassicaceous weeds, such as wild mustard (*Sinapis arvensis* L.), prior to migrating to canola plants during the bud and flowering stage (Dosdall et al., 2001; Dosdall and Moisey, 2004). Following mating, female beetles oviposit a single egg into each canola pod. Hatched larvae feed on multiple seeds within the pod. Mature larvae chew a hole through the pod and drop to the soil where pupation occurs. Adults emerge and often feed on the canola prior to seeking out overwintering sites nearby (Dosdall et al., 2001).

Currently, chemical control is used to manage CSW populations in western Canada with pyrethroid compounds being the most effective (Carcamo et al., 2005). In contrast, in Europe, two hymenopteran ectoparasitoid species *Trichomalus perfectus* (Walker) (Chalcidoidea: Pteromalidae) and *Mesopolobus morys* (Walker) (Chalcidoidea: Pteromalidae) reduce populations of CSW with *T. perfectus* often being the prevalent species. These parasitoids are not present in North America. Variable rates of parasitism have been reported between different regions within Europe; parasitism of CSW larvae by endemic parasitoids is capable of reaching 100% (reviewed in Williams, 2003). Release of the parasitoids *T. perfectus* and *M. morys* into British Columbia in 1949 (McLeod, 1951) resulted in 80-90% parasitism rate initially on CSW

(McLeod, 1953). However, these two species did not become established in North America (Gillespie et al., 2006). This classical biological control method is not used in Alberta and Saskatchewan because parasitism levels by native parasitoids on invasive CSW are too low (Dosdall et al., 2009). Despite the shifting of endemic parasitoid fauna from their native hosts onto CSW populations in Western Canada, to date parasitism by these species is not as effective as parasitism by their European counterparts. The reintroduction of *T. perfectus* and *M. morys* into Canada was suggested as part of an integrated pest management strategy to control CSW populations that are not sufficiently controlled by endemic parasitoids (Gillespie et al., 2006; Dosdall et al., 2009). A recent survey of hymenopteran larval parasitoids attacking CSW identified four dominant genera in Saskatchewan and Alberta: *Trichomalus lucidus* (Walker) (Chalcidoidea: Pteromalidae), *Necremnus tidius* (Walker) (Chalcidoidea: Eulophidae), *Chlorocytus* sp. (Chalcidoidea: Pteromalidae) and *Pteromalus* sp. (Chalcidoidea: Pteromalidae) (Dosdall et al., 2009). Although high parasitism levels of 45% were recorded with *N. tidius* within one year, the level is usually around 4-12% depending on the area (Dosdall et al., 2007). Variable parasitization rates between years have also occurs for the other parasitoids with a maximum parasitism level of 7% for *T. lucidus,* 10% for *Chlorocytus sp.*and 9% for *Pteromalus sp.* (Dosdall et al., 2009).

Wolbachia has been previously documented in CSW (Floate et al., 2011). Although weevils show genetic variation between different geographical areas (Laffin et al., 2005), only one strain of *Wolbachia* is common in both European

and North American CSW populations, therefore most likely resulted from a single ancestral infection (Floate et al., 2011).

In contrast, parasitoids of CSW have not been previously examined for the presence of *Wolbachia*. A *Wolbachia* infection in CSW-associated parasitoids has important implications for the successful use of parasitoids for biological control. For example, parthenogenesis inducing *Wolbachia* can result in higher paraisitization rates because of the generation of all-female populations of parasitoids (Brelsfoard and Dobson, 2009). In Alberta, total parasitism rates on CSW by endemic species, including parasitism by *T. lucidus*, a congeneric of a major parasitoid in Europe, average only 15% (Dosdall et al., 2009). This comparatively low rate may also be the result of difficulties encountered during host-shifting by the native parasitoid fauna onto CSW. Low rates of parasitization may be a result of *Wolbachia* infection. The presence of *Wolbachia* in *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae), and *Drosophila simulans* (Sturtevant) (Diptera: Drosophilidae) altered the rate of parasitization by *Microtonus aethiopoides* (Loan) (Hymenoptera: Braconidae), and *Leptopilina heterotoma* (Thomson) (Hymenoptera: Eucoilidae) respectively (Hsiao, 1996, Fytrou et al., 2006). The rapid movement of CSW throughout the canola growing region of Western Canada and the presence of several parasitoid species capable of exploting this growing resource presents a unique opportunity to study horizontal transfer through parasitization in a recently established host-parasitoid system.

In this study I identify and characterize *Wolbachia* strains and associated WO bacteriophages in CSW, and test for *Wolbachia* infection in four separate genera of parasitoids (*T. lucidus, N. tidius, Chlorocytus sp., Pteromalus sp.*) all found in Southern Alberta. Using material collected from infested canola pods of CSW, newly emerged parasitoids were evaluated for *Wolbachia* infection by PCR. Phylogenetic analysis was performed to establish whether *Wolbachia* infection is species-specific or if horizontal transfer of *Wolbachia* has occurred from host to parasitoid in recent evolutionary history. Phylogenetic analysis can identify the movement of *Wolbachia* between different insect hosts. In this study, *Wolbachia* and its associated phage WO in both CSW and the parasitoid *T. lucidus* were sequenced in order to identify potential horizontal transmission.

Materials and methods

Sample collection

Ceutorhynchus obstrictus and *T. lucidus* were collected from emergence cages containing canola seedpods from fields near Lethbridge in southern Alberta. Once collected whole insects were placed in 95% ethanol and stored at room temperature until processing. Additional *C. obstrictus* and associated parasitoids, *T. lucidus, N. tidus, Chlorocytus sp*., *Pteromalus sp.* were isolated from individual test tubes sealed with foam plugs, each containing 1 or 2 canola seedpods from *Brassica napus* L. and *Brassica rapa* L. collected from fields in the Lethbridge region. The tubes were checked daily and any emergent parasitoids or CSW larvae were placed in 95% ethanol until identification and DNA processing.

DNA extraction and sequencing

Genomic DNA was extracted from *C. obstrictus* using the DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA from parasitoids was extracted by phenol-chloroform extraction. Individual parasitoids were removed from ethanol fixation and dried at 60˚C prior to processing. The parasitoids were placed in a clean 1.5ml microfuge tube, submerged in liquid nitrogen for one minute, then homogenized by hand using a pellet pestle. The parasitoids were placed at 56˚C overnight after the addition of 330µl of 1X STE buffer, 60µl 10% SDS and 10µl Proteinase K (10mg/ml) (Sigma). All samples were centrifuged for 15 min (14,000 rpm) to pellet debris and the supernatant was transferred to a clean 1.5ml microfuge tube. Next, 400µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the supernatant, mixed by hand and left on ice for 10 minutes. Samples were centrifuged for 2 minutes (15,800xg), and the aqueous phase was transferred to a new microfuge tube. This step was repeated with 5 min incubation on ice prior to centrifugation and transfer of the aqueous phase to a new tube. To remove traces of phenol prior to ethanol precipitation, 400µl of chloroform:isoamyl alcohol (24:1) was added to each sample and centrifuged for 2 min (15,800xg), the aqueous phase placed in a new tube and 2 volumes of 95% ethanol and 1/10 volume of 3M sodium acetate (NaAc) was added. Samples were placed at -20˚C for 1 hour to precipitate DNA.

Samples were analyzed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) to determine total DNA concentration and purity based on its

A260/A280 ratio, making sure no contaminants such as residual phenol or ethanol were present which may interfere with downstream use of DNA. The presence of *Wolbachia* was determined by PCR amplification of the *Wolbachia* surface protein using primers wsp81f/wsp691r (Zhou et al., 1998). Typing *Wolbachia* was done using PCR products of *ftsZ* and gatB. The WO phage was typed with the orf7 gene. All primers are listed in Table 2.1. PCR products were cloned into the pCR®4-TOPO® vector and inserted into One Shot® TOP10 competent cells (TOPO TA Cloning Kit for Sequencing, Invitrogen). Three colonies were selected and the cloned gene was sequenced using a LiCor 4300 DNA analyzer with labeled primers and Thermo Sequenase labeled primer cycle sequencing kit (USB). A consensus sequence was generated for each gene prior to phylogenetic analysis.

Phylogenetic Analyses

The *ftsZ* and *gatB* DNA sequences were concatenated for each species prior to alignment. Sequences were aligned with ClustalW and modified with BioEdit v7.0.9 (Thomas et al., 1994; Hall, 1999). For all genes, the evolutionary history was inferred using the maximum parsimony (MP) methods and validated using maximum likelihood (ML) method. Phylogenetic trees using MP were generated using PAUP* version 4.0b10 (Swofford, 2003). Bootstrap values were calculated from 100 replicates using 10 random taxon addition replicates TBR branch swapping. Phylogenetic analysis using ML was conducted using PHYML (Guindon and Gascuel, 2003).Modeltest version 3.06 and the Akaike information

criterion (AIC) were used to select a DNA substitution model for each data set before ML analysis: concatenated *ftsZ* and *gatB* (GTR+Γ), *orf7* (GTR+I+Γ) (Posada and Crandall, 1998). ML heuristic searches were conducted with the GTR substitution model (estimated proportion of invariable sites, single substitution rate category). Bootstrap values were calculated from 100 replicates replicates and NNI branch swapping. Due to the lack of a suitable outgroup both trees generated are midpoint-rooted.

Quantitative PCR

In order to determine the impact of *Wolbachia* infection in CSW, *Wolbachia* density was measured. Infection density of *Wolbachia* in larvae of CSW was measured by quantitative PCR (qPCR). Using this technique, I compared the *ftsZ* gene copy number (*Wolbachia*) to the copy number of the elongation factor 1 α (*ef1a*) gene, a nuclear gene of CSW selected due to sequence availability (primers are shown in Table 2.2). Primers for EF1α were designed against EF1α of CSW (EU156572 and EU156571 consensus sequence) using Geneious 6.1.2 primer design tool to amplify a 75 bp region. qPCR was done using the 7500 Fast Real-Time PCR System (Life Technologies). Individual reactions were carried out in a MicroAmp Fast optical 96 well reaction plate (Life Technologies). Each well contained 5µl of 2x Dynamite qPCR master mix (Molecular Biology Service Unit, University of Alberta), composed of SYBR green (Molecular Probes) and platinum Taq (Invitrogen), 0.4µM of primers, and 50ng/µl of template DNA. PCR conditions were defined with an initial melting temperature of 95˚C for 5 minutes,

followed by 40 cycles of 95˚C for 15 seconds, 55˚C for 15 seconds, and 72˚C for 30 seconds. A melting curve analysis was done at the end of the PCR cycle to ensure the purity of the amplicon. All reactions for each individual insect for each primer set were performed in triplicate and averaged before analysis.

A standard curve for EF1α-75f/157R was used to determine primer efficiency and was developed with a series of five-fold dilutions of genomic DNA. Good primer efficiency (E=101%, r^2=0.99) was determined using a qPCR Efficiency Calculator (Kapa Biosystems). Primer efficiency was determined for *ftsZ* primers prior to this study (90%<E<110%) (J. Haukedal, *personal communication*). Comparisons between larval weight and *Wolbachia* number was done separately on mature third instar CSW that fed on *B. napus* or *B. rapa* using Pearson's coefficient of correlation.

Results

Initial screening of CSW and *T. lucidus* from emergence cages revealed that both insects harboured a *Wolbachia* infection. Phylogenetic analysis revealed that the *Wolbachia* strains found in CSW and *T. lucidus* were members of two separate supergroups, group A and B respectively (Figure 2.5). Phylogenetic analysis of the bacteriophage WO orf7 gene showed that the phages present in *Wolbachia* from CSW and *T. lucidus* were also evolutionarily distant (Figure 2.6).

Parasitoids that emerged from CSW in individual seedpods in test tubes were identified as members of four distinct species: *T. lucidus, N. tidus, Chlorocytus* sp., *Pteromalus* sp. One species, *N. tidus* accounted for 50% of the

emerged parasitoids, 25% were *T. lucidus*, 18.75% were *Chlorocytus* sp., and 6.25% were *Pteromalus* sp.. Sequencing results for *Wolbachia ftsZ* and *gatB* from CSW examined in this study are identical to those previously published by Floate et al. (2011) for Alberta populations of CSW.

Individual weights of mature third instar CSW larvae that emerged from *B. napus* and *B. rapa* seedpods were determined. The density of *Wolbachia* in CSW larvae was assessed by measuring the ratio of the *Wolbachia ftsZ* copy number to CSW *ef1α* copy number (Figure 2.7). *Wolbachia* copy number was not significantly different between CSW emerging from *B. napus* (8.2±3.97 *Wolbachia*/cell) and *B. rapa* (9.45±3.42 *Wolbachia*/cell) (P=0.49, Student's *t*-test). In addition, there was no significant difference between larval weights of insects that fed on *B. napus* (.0043g±.0008g) and *B. rapa* (.0037g±.0010g) (P=0.13, Student's *t*-test). *Wolbachia* copy number per gram of larval wet weight in *B. napus* (1873.1*Wolbachia*/g±.882.9) and *B. rapa* (2773.4 *Wolbachia*/g ±1331.1) also showed no significant difference (P=0.063, Student's *t*-test). There was no observed correlation between *Wolbachia* density and larval weight (Pearson r = -0.093).

Discussion

The horizontal transfer of *Wolbachia* through host-parasitoid systems has been suggested by previous phylogenetic studies of field collected insects (Werren et al., 1995; Schilthuizen and Stouthamer 1997; Vavre et al., 1999; Noda et al., 2001; Batista et al., 2010), and also has been shown to occur in laboratory conditions

(Heath et al., 1999; Huigens et al., 2004). The presence of *Wolbachia* and its associated phage WO in indigenous parasitoids which are shifting to a new introduced host (CSW) was examined here to determine if there has been horizontal transfer of *Wolbachia* between these insects under field conditions. Of all the insect species examined, *Wolbachia* was detected only in CSW and *T. lucidus*. However, phylogenetic analysis of *Wolbachia* strains in CSW and *T. lucidus* places the two *Wolbachia* strains into supergroups A and B, respectively (Figure 2.5). Bacteriophage WO strains in *Wolbachia* from CSW and *T. lucidus* were also evolutionarily distant (66.4% sequence similarity) (Figure 2.6). There is no evidence of horizontal transfer of *Wolbachia* or WO prophage between CSW (A-supergoup *Wolbachia*) and *T. lucidus* (B-supergoup *Wolbachia*). The partial sequence of *orf7* of the WO prophage of *T. lucidus* shares 94.7% sequence similarity to the WO prophage field cricket *Teleogryllus taiwanemma* (Ohmachi and Matsumura) (Orthoptera: Gryllidae). This similarity is likely due to the limited overlap between the partial orf7 gene from *T. lucidus* to that of WO prophage in *T. taiwanemma*. *Wolbachia* was not identified from any of the other three parasitoid species.

In contrast to *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) and its associated parasitoid *Diadegma insulare* Förster (Hymenoptera: Ichneumonidae), which share identical strains of both *Wolbachia* and WO phage (Batista et al., 2010, and Chapter 2a), the interaction between populations of CSW and *T. lucidus* may be too recent for the horizontal transfer of *Wolbachia* to occur. Alternatively, the *Wolbachia* in CSW may not be able to colonize a novel parasitoid host. Vavre

et al. (2003) describe three filters that must be overcome by *Wolbachia* for horizontal transfer into a new species. These are the encounter filter, which is a physical interaction between bacteria and host leading to infection, the compatibility filter, meaning that the *Wolbachia* strain must be capable of establishing itself in the germ line cells of the new host, and, lastly, the invasion filter, which is the ability of *Wolbachia* to be maintained in a population. The lack of horizontal transfer between CSW and *T. lucidus* may be due to the inability of the *Wolbachia* strain to establish itself in the germ line of the new host, or if it does, it may be selected out as a result of possible fitness costs, or reproductive incompatibility. Further study is required to identify whether one or more of these factors are restricting transfer of *Wolbachia* between CSW and these parasitoid species.

One *Wolbachia* strain is found in all populations of CSW examined, which most likely reflects a single infection in an ancestral population (Floate et al., 2011). This may represent a specialization of this *Wolbachia* strain for this taxonomic group. However, a comparison of *Wolbachia* strains found in *Ceutorhynchus* species (*C. neglectus* (Blatchley) (Coleoptera: Curculionidae) and *C. subpubescens* (LeConte) (Coleoptera: Curculionidae)) native to Canada showed no similarity to the CSW *Wolbachia* strain (Floate et al., 2011). Likely this is a result of different geographic origins of the *Ceutorhynchus* species examined. If the *Wolbachia* strain infecting CSW is specialized to its host, then horizontal transfer would seem unlikely as a result of *Wolbachia*-host

coadaptation. The potential for horizontal transfer between CSW and its associated host-specific parasitoids in Europe has not been determined.

Unlike DBM, in which *Wolbachia* infection rates vary between populations, (from as high as 40% infection rate to some populations not having any detectable *Wolbachia* infection, Delgado and Cook, 2009), all populations of CSW that have been examined are completely infected with *Wolbachia* (Floate et al., 2011, this study). In all populations of CSW, *Wolbachia* is vertically transmitted with 100% fidelity, meaning that CSW is stably infected with its host. The *Wolbachia* density of emerged CSW from *B. napus* and *B. rapa* pods was examined in order to determine if there was any relationship between host plant, larval weight and *Wolbachia* density. Although *Wolbachia* density was variable (3-15 bacterial gene copies per *ef1α* for *B. napus* and 5-17 bacterial gene copies per *ef1α* for *B. rapa*) for CSW larvae from both host plants; there was no significant difference in mean *Wolbachia* density or larval weight between *B. napus* and *B. rapa*. In *D. simulans*, in which *Wolbachia* is stably infected and caused complete CI (J. Haukedal, *personal communication*) reported an average copy number of 6.5 *Wolbachia* number per host cell for males, and 10.5 copy number for females. This suggests that the *Wolbachia* infecting CSW has a relatively high density of *Wolbachia*, and lack of horizontal transfer is not due to low *Wolbachia* titer. There was also no observed correlation between larval weight and *Wolbachia* density.

Wolbachia density in insects feeding on two plant species has not previously been measured. There was no observed difference in overall nutrition of CSW

larvae feeding on *B. napus* or *B. rapa,* nor was a difference in *Wolbachia* density observed. In addition, there is no correlation between the weight of the larvae and *Wolbachia* copy number, supporting previous evidence that nutritional variation has no effect on *Wolbachia* density (Unkless et al., 2009). However this is dependent on the presence of antioxidants in the diet which does have a positive effect on *Wolbachia* density. In *D. simulans Wolbachia* density doubles as a result of glutathione availability (J. Haukedal, *personal communication*).

This study presents the only quantitative data on *Wolbachia* density in weevil larvae. Studies examining adults of rice weevil *Sitophilus oryzae* revealed variable tissue tropism showing a higher *Wolbachia* density in the reproductive organs than in other tissues (Heddi et al., 1999). Tissue distribution was not examined in this study and *Wolbachia* density represents the average *Wolbachia* population in all tissues within an individual. In addition the sex of each CSW larvae was not determined in this experiment and variability of the *Wolbachia* copy number and copy number per gram of larval weight for *B. napus* and *B. rapa* may be explained by differences between *Wolbachia* density in males and females, with males having a lower overall *Wolbachia* density (Tortosa et al., 2010; J. Haukedal, *personal communication*).

Wolbachia density has been shown to be important in conferring antiviral protection to its host in *D. simulans* (Osborne et al., 2009; Osborne et al., 2012) and *Aedes aegypti* (Moreira et al., 2009). It is currently unknown if the role of the antiviral ability conferred by *Wolbachia* on its host is dependent on the distribution within certain tissues (Osborne et al., 2012). Whether the *Wolbachia*

found in all populations in CSW offer antiviral protection or possibly present some protection to parasitoids has not been determined. The data presented here provides a starting point for future studies investigating changes in *Wolbachia* density throughout development of CSW.

Although host-parasitoid systems can provide a route for the horizontal transfer of *Wolbachia*, the lack of horizontal transfer between CSW and *T. lucidus* illustrates the complexity of the symbiotic relationship. Compatibility between a *Wolbachia* strain and its host and a longer evolutionary timescale are likely needed for horizontal transfer to occur.

The ability for *Wolbachia* to determine the success of a parasitoid either through induced reproductive phenotypes (Brelsfoard and Dobson, 2009) or by influencing the host immune response to parasitization (Hsiao, 1996; Fytrou et al., 2006) may explain the low level of parasitism of native parasitoids in introduced CSW populations. The role of *Wolbachia* in the success of this invasive host and host-switching parasitoids needs to be examined further.

Tables

Table 2.1. Primers used in this study for PCR amplification of different *Wolbachia* genes.

Gene	Product	Primer Name	Sequence 5'-3'	References
gatB	Glutamyl-tRNA (Gln) amidotransferase, subunit B	gatB_F1	GAKTTAAAYCGYGCAGGBGTT	Baldo et al. 2006
		gatB_R1	TGGYAAYTCRGGYAAAGATGA	
ftsZ	Cell division protein	ftsZ_F1	ATYATGGARCATATAAARGATAG	
		ftsZ_R1	TCRAGYAATGGATTRGATAT	
		ftsZunif	GG(CT)AA(AG)GGTGC(AG)GCAGAAGA	Lo et al. 2002
		ftsZunir	ATC(AG)AT(AG)CCAGTTGCAAG	
groEL	heat shock protein HSP60	WgroBF2	CAG AGG TYA CAA AGG ATG GC	Baldo et al. 2006b
		WgroBR2	AAT GCT TCA CCTTCA ACA TCT	
wsp	*Wolbachia* surface protein	81F	TGGTCC AAT AAGTGA TGA AGA AAC	Zhou et al. 1998
		136F	TGAAA TTT TAC CTC TTT TC	
		522R	ACC AGC TTT TGC TTGATA	
		691R	AAA AAT TAA ACGCTA CTC CA	
orf7	Minor capsid protein	phgWOF	CCCACATGAGCCAATGACGTCTG	Masui et al. 2000
		phgWOR	CGTTCGCTCTGCAAGTAACTCCATTAAAAC	

Table 2.2. Primers used in this study for qPCR amplification

Gene	Product	Primer Name	Sequence 5'-3'	References
eF1a	Elongation factor 1 alpha	157R -	TAA TTA CCT GGA GGG GAA GA	This study
		75F -	ATG CCT TAT TGA AGC TTT GG	
ftsZ	Cell division protein	wRiFtsZ-F	TGT CTA TTG ATC TTA GTC TGC C	J. Haukedal (unpublished)
		wRiFtsZ-R	GTT ATT CACA GCA TTT CCA C	

Figures

Figure 2.1: Rearing cage setup for P*lutella xylostella* and *Diadegma insulare*. Insects were reared on *Brassica napus* cv. Q2 plants (not shown here) contained in each cage.

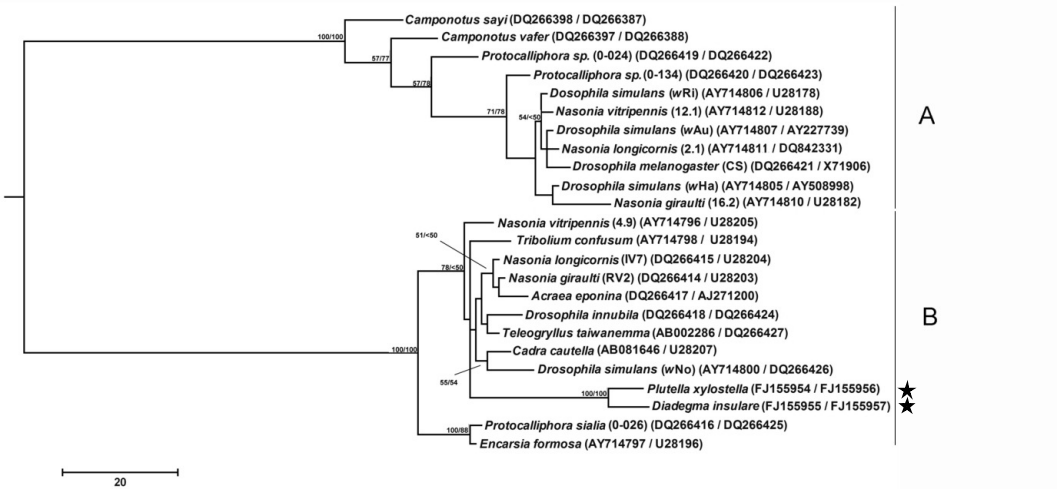


Figure 2.2. Midpoint rooted phylogenetic tree of *Wolbachia* found in *Plutella xylostella* and *Diadegma insulare* based on maximum parsimony for the concatenated alignment of *groEL* and *ftsZ*. Name of the host arthropod species is followed by accession number of *groEL* and *ftsZ*, in this order. The A- and B-supergroup *Wolbachia* clades are labeled. The position of *P. xylostella* and *D. insulare* within supergroup B are indicated by stars. Bootstrap values for nodes are listed for maximum parsimony and maximum likelihood analyses.

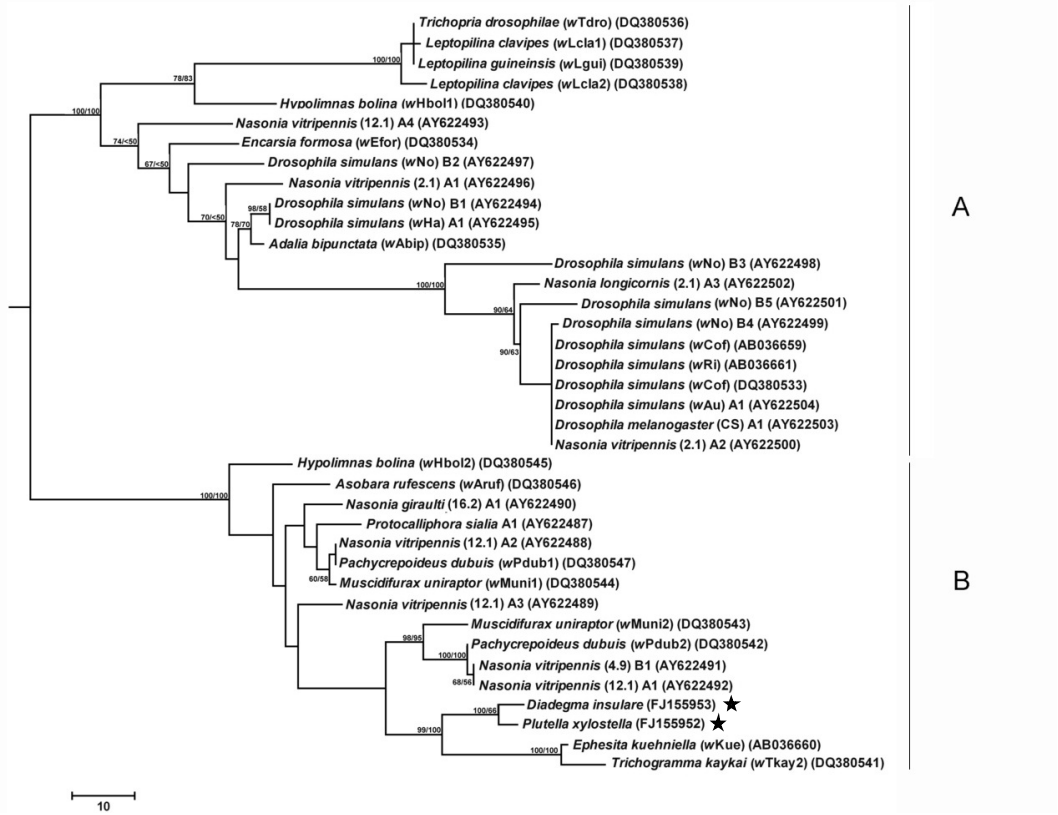


Figure 2.3. Midpoint rooted phylogenetic tree of bacteriophage WO found in *Plutella xylostella* and *Diadegma insulare* based on maximum parsimony for *orf7*. Name of the host arthropod species followed by accession number. The position of *P. xylostella* and *D. insulare* within supergroup B are indicated by stars. Bootstrap values for nodes are listed for maximum parsimony and maximum likelihood analyses.

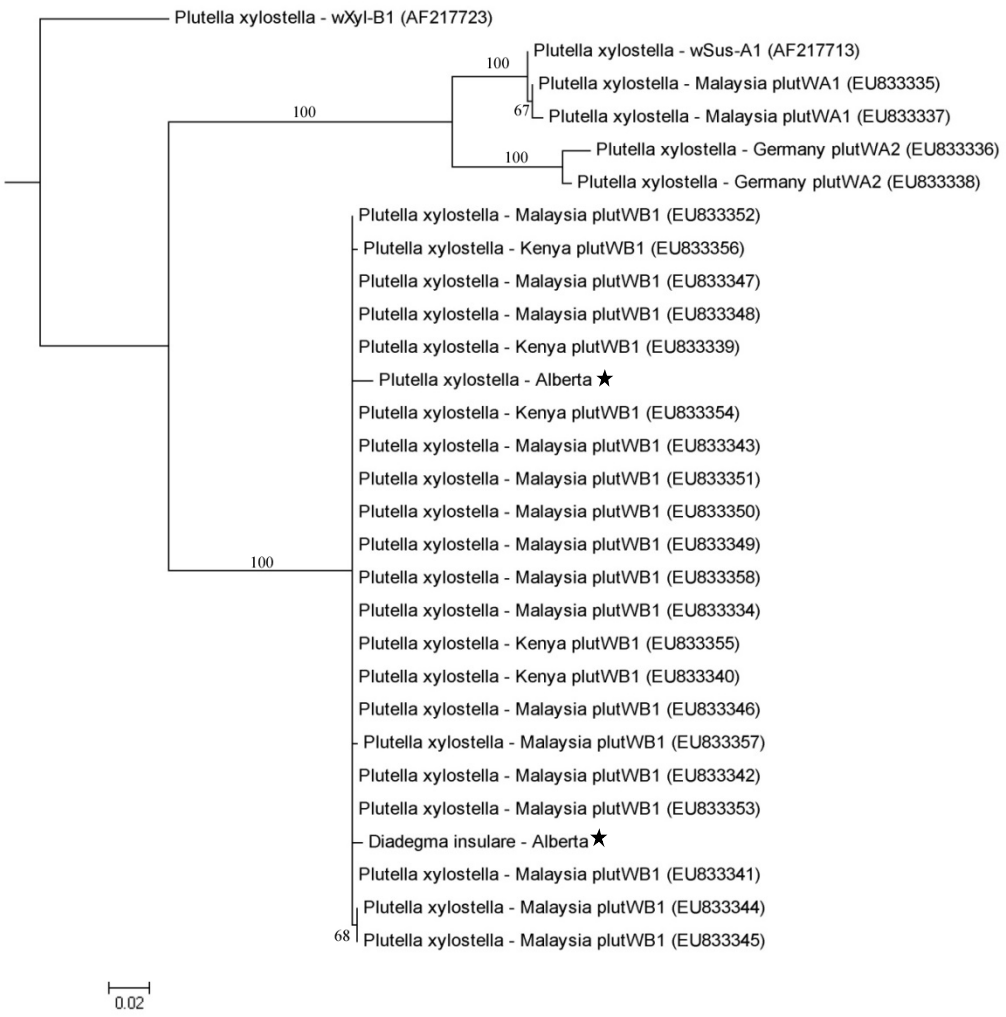


Figure 2.4. Maximum likelihood phylogeny of *Wolbachia* in different populations of *Plutella xylostella* based on *wsp.* Tree is midpoint rooted with bootstrap values listed for branches. Name of the host arthropod species and its location are followed by accession number. Sequence data for *Wolbachia wsp* in *P. xylostella* and *Diadegma insulare* from Alberta populations have not been submitted to Genbank.

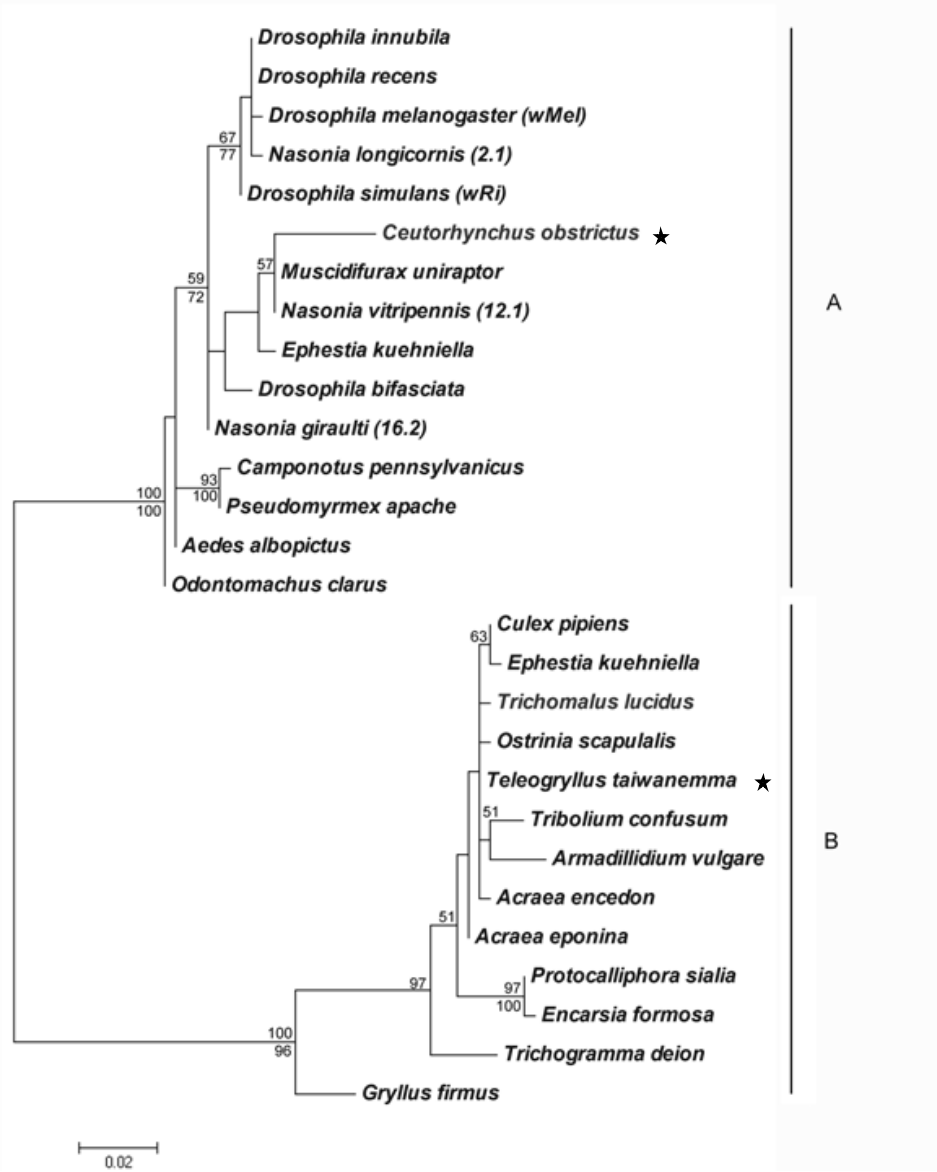


Figure 2.5. Maximum parsimony phylogenetic tree of *Wolbachia* found in *Ceutorhynchus obstrictus* and *Trichomalus lucidus* based on the concatenated alignment of *gatB* and *ftsZ*. . The A- and B- supergroup *Wolbachia* clades are labeled. The position of *C. obstrictus* and *T. lucidus* within supergroup B are indicated by stars. Tree is midpoint rooted with bootstrap values for nodes are listed for maximum parsimony (above line) and maximum likelihood (below line) analyses.

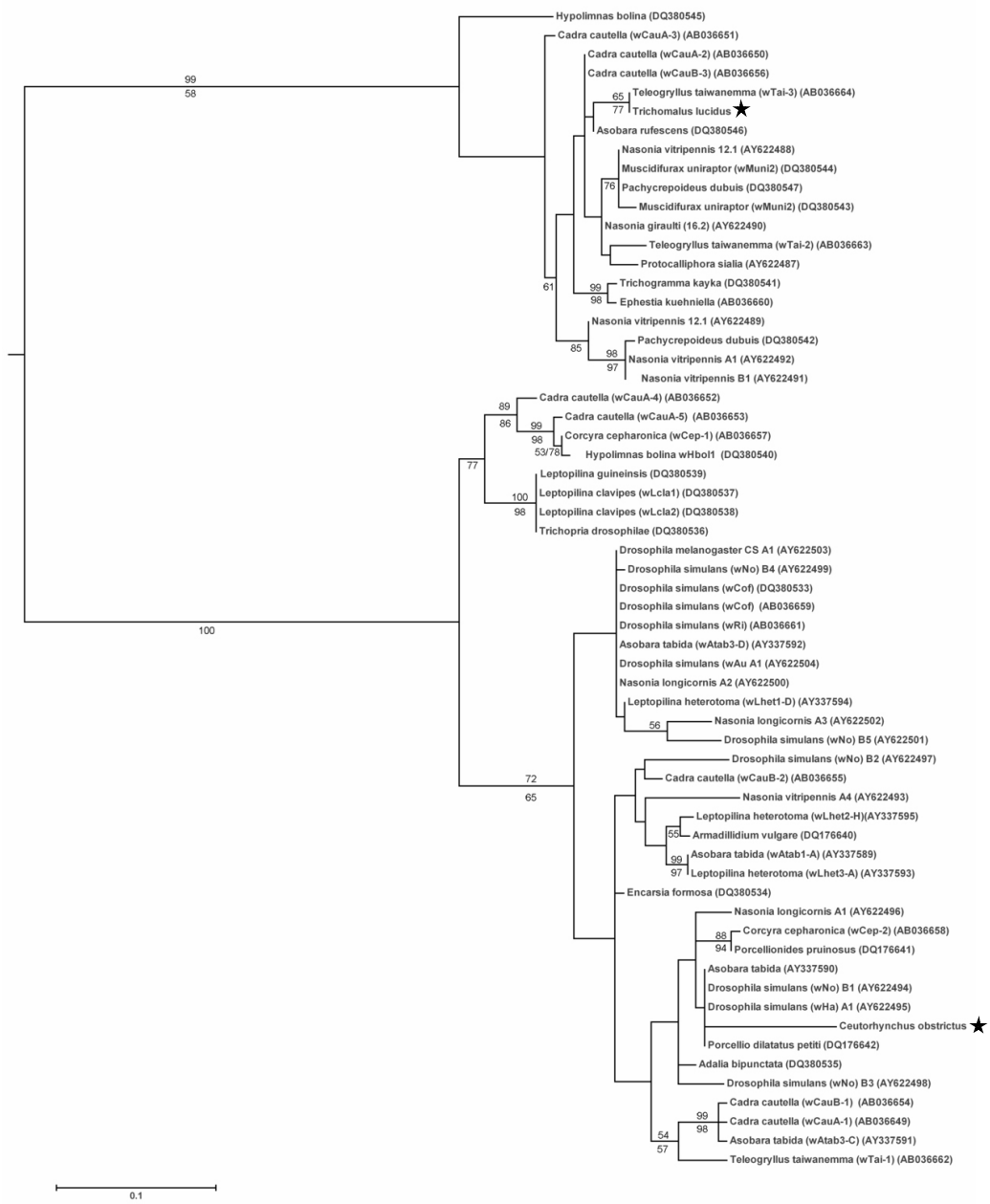

Hypolimnas bolina (DQ380545)
Cadra cautella (wCauA-3) (AB036651)
Cadra cautella (wCauA-2) (AB036650)
Cadra cautella (wCauB-3) (AB036656)
Teleogryllus taiwanemma (wTai-3) (AB036664)
Trichomalus lucidus
Asobara rufescens (DQ380546)
Nasonia vitripennis 12.1 (AY622488)
Muscidifurax uniraptor (wMuni2) (DQ380544)
Pachycrepoideus dubuis (DQ380547)
Muscidifurax uniraptor (wMuni2) (DQ380543)
Nasonia girault (16.2) (AY622490)
Teleogryllus taiwanemma (wTai-2) (AB036663)
Protocalliphora sialia (AY622487)
Trichogramma kayka (DQ380541)
Ephestia kuehniella (AB036660)
Nasonia vitripennis 12.1 (AY622489)
Pachycrepoideus dubuis (DQ380542)
Nasonia vitripennis A1 (AY622492)
Nasonia vitripennis B1 (AY622491)
Cadra cautella (wCauA-4) (AB036652)
Cadra cautella (wCauA-5) (AB036653)
Corcyra cepharonica (wCep-1) (AB036657)
Hypolimnas bolina wHbol1 (DQ380540)
Leptopilina guineinsis (DQ380539)
Leptopilina clavipes (wLcla1) (DQ380537)
Leptopilina clavipes (wLcla2) (DQ380538)
Trichopria drosophilae (DQ380536)
Drosophila melanogaster CS A1 (AY622503)
Drosophila simulans (wNo) B4 (AY622499)
Drosophila simulans (wCof) (DQ380533)
Drosophila simulans (wCof) (AB036659)
Drosophila simulans (wRi) (AB036661)
Asobara tabida (wAtab3-D) (AY337592)
Drosophila simulans (wAu A1 (AY622504)
Nasonia longicornis A2 (AY622500)
Leptopilina heterotoma (wLhet1-D) (AY337594)
Nasonia longicornis A3 (AY622502)
Drosophila simulans (wNo) B5 (AY622501)
Drosophila simulans (wNo) B2 (AY622497)
Cadra cautella (wCauB-2) (AB036655)
Nasonia vitripennis A4 (AY622493)
Leptopilina heterotoma (wLhet2-H)(AY337595)
Armadillidium vulgare (DQ176640)
Asobara tabida (wAtab1-A) (AY337589)
Leptopilina heterotoma (wLhet3-A) (AY337593)
Encarsia formosa (DQ380534)
Nasonia longicornis A1 (AY622496)
Corcyra cepharonica (wCep-2) (AB036658)
Porcellionides pruinosus (DQ176641)
Asobara tabida (AY337590)
Drosophila simulans (wNo) B1 (AY622494)
Drosophila simulans (wHa) A1 (AY622495)
Ceutorhynchus obstrictus
Porcellio dilatatus petiti (DQ176642)
Adalia bipunctata (DQ380535)
Drosophila simulans (wNo) B3 (AY622498)
Cadra cautella (wCauB-1) (AB036654)
Cadra cautella (wCauA-1) (AB036649)
Asobara tabida (wAtab3-C) (AY337591)
Teleogryllus taiwanemma (wTai-1) (AB036662)
0.1

Figure 2.6. Maximum parsimony phylogenetic tree of WO prophage of *Wolbachia* found in *Ceutorhynchus obstrictus* and *Trichomalus lucidus* based on the concatenated alignment of orf7. The position of *C. obstrictus* and *T. lucidus* within supergroup B are indicated by stars. Tree is midpoint rooted with bootstrap values for nodes are listed for maximum parsimony (above line) and maximum likelihood (below line) analyses.

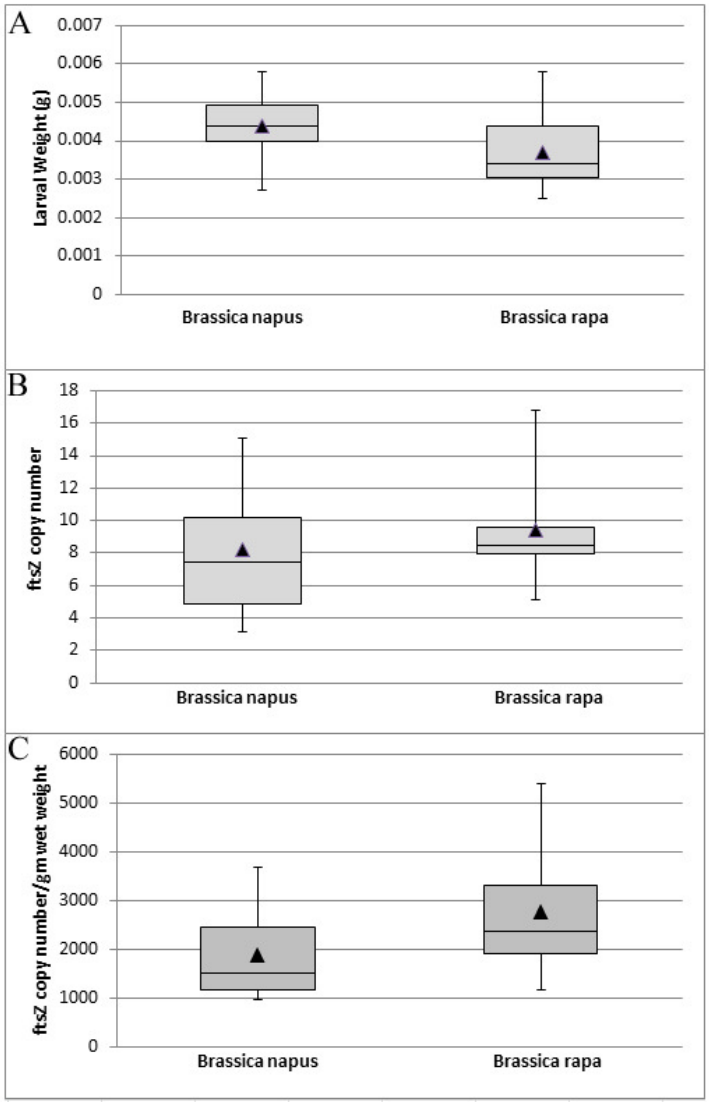


Figure 2.7. (A) Larval weight was measured from unsexed individual ethanol-fixed emerged CSW. (B) Relative *Wolbachia* copy number in CSW from *B. napus* (n=12) and *B. rapa* (n=12) measured by qPCR. Copy number of *Wolbachia* encoded *ftsZ* was compared to the copy number of elongation factor 1 α (*ef1a*) gene (a host nuclear gene) in order to determine the relative *Wolbachia* density of individual CSW larvae. (C) *Wolbachia* density per gram of larval wet weight.

References

Batista, PD. Keddie, BA; Dosdall, LM.; Harris, HL. 2010. Phylogenetic placement and evidence for horizontal transfer of *Wolbachia* in *Plutella xylostella* (Lepidoptera: Plutellidae) and its parasitoid, *Diadegma insulare* (Hymenoptera: lchneumonidae). The Canadian Entomologist 142(1);57-64

Baldo, L., Bordenstein, S., Wernegreen, J.J., Werren, J.H. 2006. Widespread recombination throughout *Wolbachia* genomes. Molecular Biology and Evolution, 23: 437-449.

Baldo, L., Lo, N., Werren, J.H. 2005. Mosaic nature of the *Wolbachia* surface protein. Journal of Bacteriology, 187:5406-5418.

Bordenstein, S.R., Wernegreen, J.J. 2004. Bacteriophage flux in endosymbionts (*Wolbachia*): infection frequency, lateral transfer, and recombination rates. Molecular Biology and Evolution, 21:1981-1991.

Braig, H.R., Zhou, W., Dobson, S.L., O'Neill, S.L. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. Journal of Bacteriology, 180:2373-2378.

Braun, L., O. Olfert, J. Soroka, P. Mason, and L.M. Dosdall. 2004. Diamondback moth biocontrol activities in Canada. Pages 144-146 *In*: A.A. Kirk, and D. Bordat (eds.): Improving Biocontrol of *Plutella xylostella*. Proceedings of the International Symposium, 21-24 October 2002, Montpellier, France.

Brelsfoard CL, Dobson SL (2009) *Wolbachia*-based strategies to control insect pests and disease vectors. Asia-Pacific Journal of Molecular Biology and Biotechnology. 17: 55–63.

Cárcamo, H.A., L. Dosdall, M. Dolinski, O. Olfert, and J.R. Byers. 2001. The cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Coleoptera: Curculionidae) - a review. Journal of the Entomological Society of British Columbia, 98:201-210.

Cárcamo, H. A., L. M. Dosdall, D. Johnson, and O. Olfert. 2005. Evaluation of foliar and seed treatments for control of the cabbage seedpod weevil (Coleoptera: Curculionidae) in canola. The Canadian Entomologist, 137: 476–487.

Casiraghi, M., Bordenstein, S.R., Baldo, L., Lo, N., Beninati, T., Wernegreen, J.J., Werren, J.H., Bandi, C. 2005. Phylogeny of *Wolbachia pipientis* based on *gltA*, *groEL* and *ftsZ* gene sequences: clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the *Wolbachia* tree. Microbiology, 151:4015-4022.

Delgado, A.M., Cook, J.M. 2009. Effects of a sex-ratio distorting endosymbiont on mtDNA variation in a global insect pest. BMC Evolutionary Biology, 3;9:49.

Dosdall, L.M., G.A.P. Gibson, O.O. Olfert, and P.G. Mason. 2009. Responses of Chalcidoidea (Hymenoptera) parasitoids to invasion of the cabbage seedpod weevil (Coleoptera: Curculionidae) in western Canada. Biological Invasions. 11:109-125.

Dosdall, L.M., Mason, P.G., Olfert, O., Kaminski, L., Keddie, B.A. 2004. The origins of infestations of diamondback moth, *Plutella xylostella* (L.), in canola in western Canada. *In* The management of diamondback moth and other crucifer pests. *Edited by* N.M. Endersby, P.M., Ridland. Proceedings of the Fourth International Workshop, 26-29 November 2001. Melbourne, Australia: Department of Natural Resources and Environment. pp. 95-100.

Dosdall, L.M., Soroka, J.J., and Olfert, O.O. 2011. The Diamondback Moth in Canola and Mustard: Current Pest Status and Future Prospects. Prairie Soils and Crops, 4; 66-76.

Dosdall, L.M., and D.W.A. Moisey. 2004. Developmental biology of the cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Coleoptera: Curculionidae), in spring canola, *Brassica napus*, in western Canada. Annals of the Entomological Society of America 97: 458-465.

Dosdall, L. M., D. Moisey, H. Cárcamo, and R. Dunn. 2001. Cabbage seedpod weevil fact sheet. Alberta Agriculture, Food and Rural Development, Edmonton, AB, Canada.

Dosdall LM, Harker KN, O'Donovan JT, Blackshaw RE, Kutcher HR, Gan Y, Johnson EN. 2012. Crop sequence effects on root maggot (Diptera: Anthomyiidae:

Delia spp.) infestations in canola. Journal of Economic Entomology, 105(4):1261-7.

Dosdall, L.M., R.M. Weiss, O. Olfert, and H.A. Cárcamo. 2002. Temporal and geographical distribution patterns of cabbage seedpod weevil (Coleoptera: Curculionidae) in canola. The Canadian Entomologist. 134:403-418.

Dosdall, L. M., G.A.P. Gibson, O. Olfert, B. A. Keddie, and B. J. Ulmer. 2007. Contributions to the life history, host range, and distribution of *Necremnus tidius* (Hymenoptera: Eulophidae). Annals of the Entomological Society of America. 100: 861–868.

Floate KD, Coghlin PC, Dosdall L. 2011. A test using *Wolbachia* bacteria to identify Eurasian source populations of cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Marsham), in North America. Environmental Entomology, 40(4):818-23.

Fytrou, A., Schofield, P.G., Kraaijeveld, A.R., Hubbard, S.F. 2006. *Wolbachia* infection suppresses both host defence and parasitoid counter-defence. Proceedings of the Royal Society B: Biological Sciences, 273:791-796.

Gadagkar SR, Rosenberg MS, Kumar S. 2005. Inferring species phylogenies from multiple genes: concatenated sequence tree versus consensus gene tree. Journal of experimental zoology. Part B, Molecular and developmental evolution, 304(1):64-74.

Gavotte, L., Henri, H., Stouthamer, R., Charif, D., Charlat, S., Boulétreau, M., Vavre, F. 2007. A survey of the bacteriophage WO in the endosymbiotic bacteria *Wolbachia*. Molecular Biology and Evolution, 24:427-435.

Gillespie, D.R., P.G. Mason, L.M. Dosdall, P. Bouchard, and G.A.P. Gibson. 2006. Importance of long-term research in classical biological control: An analytical review of a release against the cabbage seedpod weevil in North America. Journal of Applied Entomology. 130:401-409.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology, 52(5):696-704.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41:95-98.

Heath BD, Butcher RD, Whitfield WG, Hubbard SF. 1999. Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. Current Biology, 9(6):313-6.

Heddi A, Grenier AM, Khatchadourian C, Charles H, Nardon P. 1999. Four intracellular genomes direct weevil biology: nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*. Proceedings of the National Academy of Sciences USA, 96(12):6814-9.

Hedges, L.M., Brownlie, J.C., O'Neill, S.L., Johnson, K.N. 2008. *Wolbachia* and virus protection in insects. Science, 322: 702

Holden, P.R., Brookfield, J.F., Jones, P. 1993. Cloning and characterization of an ftsZ homologue from a bacterial symbiont of *Drosophila melanogaster*. Molecular and General Genetics, 240:213-220.

Hsaio, T.H. 1996. Studies of interactions between alfalfa weevil strains, *Wolbachia* endosymbionts and parasitoids. *In* The Ecology of Agricultural Pests: Biochemical Approaches. *Edited by* W.O.C. Symondson and J.E. Liddell. Chapman and Hall, London, UK. pp. 51-71.

Huigens, M. E., de Almeida, R. P., Boons, P. A. H., Luck, R. F. & Stouthamer, R. 2004. Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. Proceedings of the Royal Society B: Biological Sciences 271, 509–515.

Jeyaprakash, A., Hoy, M.A. 2000. Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Molecular Biology, 9:393-405.

Koch, M. Al-Shehbaz I.A., Mummenhoff, K. 2003. Molecular systematics, evolution, and population biology in the mustard family (Brassicaceae). Annals of the Missouri Botanical Garden, 90: 131-171.

Laffin, R.D., L.M. Dosdall, and F.A.H. Sperling. 2005. Population structure of the cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Marsham) (Coleoptera: Curculionidae): Origins of North American introductions. Environmental Entomology 34:504-510.

Lamb, R.J. 1989. Entomology of oilseed *Brassica* crops. Annual Review of Entomology, 34:211-29.

Lo, N., Casiraghi, M., Salati, E., Bazzocchi, C., Bandi, C. 2002. How many *Wolbachia* supergroups exist? Molecular Biology and Evolution, 19:341-346.

Masui, S., Kamoda, S., Sasaki, T., Ishikawa, H. 2000. Distribution and evolution of bacteriophage WO in *Wolbachia*, the endosymbiont causing sexual alterations in arthropods. Journal of Molecular Evolution, 51:491-497.

Masui, S., Sasaki, T., Ishikawa, H. 1997. groE-homologous operon of *Wolbachia*, an intracellular symbiont of arthropods: a new approach for their phylogeny. Zoological Science, 14:701-706.

McLeod JH, 1951. Biological control investigations in British Columbia. Proceedings of the Entomological Society of British Columbia. 47, 27–36.

McLeod JH, 1953. Notes on the cabbage seedpod weevil *Ceutorhynchus assimilis (*Payk.) (Coleoptera: Curculionidae), and its parasites. Proceedings of the Entomological Society of British Columbia. 49, 11–18.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O'Neill SL. 2009. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. Cell, 139(7):1268-78.

Noda H, Miyoshi T, Zhang Q, Watanabe K, Deng K, Hoshizaki S. 2001. *Wolbachia* infection shared among planthoppers (Homoptera: Delphacidae) and their endoparasite (Strepsiptera: Elenchidae): a probable case of interspecies transmission. Molecular Ecology, 10(8):2101-6.

Osborne SE, Leong YS, O'Neill SL, Johnson KN. 2009. Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. PLoS Pathogens 5:e1000656.

Osborne SE, Iturbe-Ormaetxe I, Brownlie JC, O'Neill SL, Johnson KN. 2012. Antiviral protection and the importance of *Wolbachia* density and tissue tropism in *Drosophila simulans*. Applied and Environmental Microbiology, 78(19):6922-9.

Posada, D., Crandall, K.A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics, 14: 817-818.

Putnam, L.G. 1978. Diapause and cold hardiness in *Microplitis plutellae*, a parasite of the larvae of the diamondback moth. Canadian Journal of Plant Science, 58: 911-913.

Rigaud, T., Juchault, P. 1995. Success and failure of horizontal transfers of feminizing *Wolbachia* endosymbionts in woodlice. Journal of Evolutionary Biology, 8: 249-255.

Ros, V.I., Fleming, V.M., Feil, E.J., Breeuwer, J.A. 2009. How diverse is the genus *Wolbachia*? Multiple-gene sequencing reveals a putatively new *Wolbachia* supergroup recovered from spider mites (Acari: Tetranychidae). Applied and Environmental Microbiology, 75:1036-1043.

Sarfraz, M., Keddie, B.A. 2005. Conserving the efficacy of insecticides against *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). Journal of Appled Entomology, 129: 149-157.

Sarfraz, M., Dosdall, L.M., Keddie, B.A. 2006. Diamondback moth–host plant interactions: Implications for pest management. Crop Protection, 25: 625-639.

Sarfraz, M., Keddie, A.B., Dosdall, L.M. 2005. Biological control of the diamondback moth, *Plutella xylostella*: A review. Biocontrol Science and Technology, 15: 763-789.

Schilthuizen M, Stouthamer R. 1997. Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. Proceedings of the Royal Society of London. Series B, 264(1380):361-6.

Soroka, J.J., and L.M. Dosdall. 2011. Coping with root maggots in prairie canola crops. Prairie Soils and Crops 4: 24-31.

Sintupachee, S., Milne. J.R., Poonchaisri, S., Baimai, V., Kittayapong, P. 2006. Closely related *Wolbachia* strains within the pumpkin arthropod community and the potential for horizontal transmission via the plant. Microbial Ecology, 51:294-301.

Smith, D.B., Sears, M.K. 1982. Evidence for dispersal of diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), into southern Ontario. Proceedings of the Entomological Society of Ontario, 113:21–27.

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Talekar, N.S., Shelton, A.M., 1993. Biology, ecology, and management of the diamondback moth. Annual Review of Entomology, 38: 275–301.

Teixeira, L., Ferreira, A., Ashburner, M. 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biology, 6: 2753-2763

Tortosa P, Charlat S, Labbé P, Dehecq J-S, Barré H, Weill M. 2010. *Wolbachia* age-sex-specific density in *Aedes albopictus*: a host evolutionary response to cytoplasmic incompatibility? PLoS ONE, 5(3): e9700.

Unckless RL, Boelio LM, Herren JK, Jaenike J. 2009. *Wolbachia* as populations within individual insects: causes and consequences of density variation in natural populations. Proceedings of the Royal Society of London. Series B, 276(1668):2805-11.

Van Meer, M.M., Witteveldt, J., Stouthamer, R. 1999. Phylogeny of the arthropod endosymbiont *Wolbachia* based on the wsp gene. Insect Molecular Biology,

8:399-408.

Vavre F, Fouillet P, Fleury F. 2003. Between- and within-host species selection on cytoplasmic incompatibility-inducing *Wolbachia* in haplodiploids. Evolution, 57(2):421-7.

Vavre, F., Fleury, F., Lepetit, D., Fouillet, P., Bouletreau, M. 1999. Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. Molecular Biology and Evolution, 16:1711-1723.

Werren, J.H., Zhang, W., Guo, L.R. 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proceedings of the Royal Society B: Biological Sciences, 261:55-63.

West, S.A., Cook, J.M., Werren, J.H., Godfray, H.C. 1998. *Wolbachia* in two insect host-parasitoid communities. Molecular Ecology, 7:1457-1465.

Williams, I. H. 2003. Parasitoids of cabbage seed weevil, pp. 97–112. In D. V. Alford (ed.), Biocontrol of oilseed rape pests. Blackwell Publishing, Oxford, United Kingdom.

Zhao, J.Z., Collins, H.L., Li, Y.X., Mau, R.F.L., Thompson, G.D., Hertlein, M., Andaloro, J.T., Boykin, R., Shelton, A.M. 2006. Monitoring of diamondback moth (Lepidoptera: Plutellidae) resistance to spinosad, indoxacarb, and emamectin benzoate. Journal of Economic Entomology, 99:176-181.

Zhou, W., Rousset, F., O'Neil, S. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proceedings of the Royal Society B: Biological Sciences, 265:509-515.

Chapter 3

Horizontal transfer of Bacteriophage WO

For the *Wolbachia* typed in the previously studied host-parasitoid systems an associated bacteriophage was identified by sequencing the *orf7* gene, encoding the minor capsid protein. Unfortunately, the sequenced genome for *Wolbachia* and its associated bacteriophage are not available for any of the *Wolbachia* strains examined in this thesis. Complete genomes for *Wolbachia* infecting *Drosophila simulans* and *D. melanogaster* however, provide a model system for the study of the biology and diversity of the bacteriophage infecting *Wolbachia*.

Viruses that infect prokaryotes, known as bacteriophages, or just phages, constitute the most abundant biological entities on earth with an estimated phage population size of $\geq 10^{30}$ in marine systems (Chibani-Chennoufi et al., 2004). The tailed bacteriophages make up the majority of the described bacterial viruses, with individual phages found to infect bacteria in every order (Maniloff and Ackermann, 1998). The classification of bacteriophages is based mainly on morphological and nucleic acid characteristics and at present includes 7 orders, 96 families and 420 genera (King et al., 2012). Tailed bacteriophages with dsDNA belong to the order *Caudovirales*, containing the families *Myoviridae* (contractile tails), *Siphoviridae* (long non-contractile tails) and *Podovirdiae* (short non contractile tails) (Maniloff and Ackermann, 1998). This order contains 96% of all described bacteriophages with 60% being assigned to one of the above families (Maniloff and Ackermann, 1998; Glazko et al., 2007).

The work presented here (including data generated and data analysis) was carried out by the author. This data has been published in Biliske et al., 2011. BMC Microbiology, 11:251

The bacteriophage lifecycle begins by the binding of a phage structure, such as tail fibers, to a receptor on the surface of its bacterial host. This contact is followed by an absorption step where the phage genome is transferred/injected into the cell. This genetic material can either be integrated into the bacterial genome or remain separate (reviewed by Weinbauer, 2004). Depending on the phage, the lifecycle can continue through a lytic cycle, where phage production occurs in the cell and is followed by cell lysis and release of phage particles. Alternatively, a lysogenic or temperate cycle can occur where the phage genome will reside dormant in the bacterial host, becoming what is known as a prophage (Ackermann and DuBow, 1987). In this case, the prophage will replicate with its host chromosome until it is induced to switch to a lytic cycle if it is a functional prophage. Prophages which are non-functional, and therefore unable to complete their replication cycle, may retain some functional genes (Casjens, 2003).

The presence of integrated prophages contributes to genome diversification in bacteria and accounts for a substantial amount of the total genomic DNA present in any given host (e.g. 13% of the *w*Ri genome belongs to prophages). The impact of phage DNA on bacterial evolution has been studied extensively in pathogenic bacteria where virulence or fitness factors of temperate bacteriophages are introduced into the host genome though horizontal gene transfer (Brüssow et al., 2004). An example of genome diversification due to horizontal gene transfer is seen between enterohemorrhagic *Escherichia coli* O157:H7 strains (Ohnishi et al., 2002), or between the pathogenic *E. coli* O157 and laboratory strains of *E. coli* K-12 (Hayashi et al., 2001). The pathogenic and laboratory strains share a

chromosome backbone of 4.1Mb. However, about half of the strain-specific sequences of the pathogenic O157 originate from different integrated prophages (Hayashi et al., 2001). Among strains of O157, which share a conserved bacterial chromosome, prophages account for much of the strain-to-strain differences (Ohnishi et al., 2002). Prophage regions on the bacterial chromosome of *E. coli* O157:H7 act as sites for large genome rearrangement through homologous recombination (Iguchi et al., 2006).

Although sequence similarity between prophages that have different host ranges is rare, conserved genome architecture often occurs between morphotypes (Hatfull, 2008). For example the *Siphoviridae*, a group which includes the *Wolbachia* WO phages, require a basic set of genes encoding structural proteins (head, tail, tail fibers), DNA replication and packaging, and proteins involved in cell lysis (e.g. holin) (Brüssow and Hendrix, 2002). Like bacterial chromosomes, phage genomes undergo homologous recombination. Although the organization of phage genes may be conserved between different bacteriophages, a mosaic genome structure results from the exchange of a genetic region from one phage genome into another (Brüssow and Hendrix, 2002; Brüssow et al., 2004). These hybrid prophages can still be fully functional if these exchanged genetic regions, or modules, carry out the same biological function (Botstein, 1980).

Temperate bacteriophages infecting *Wolbachia*, named WO, were discovered by Masui et al. (2000), in the insect *Teleogryllus taiwanemma* (*w*Tai). He identified seven open reading frames (orfs) belonging to the prophage in this strain. *Wolbachia* belonging to *Corcyra cepharonica* (*w*Cep), *Drosophila*

simulans (*w*Cof, *w*Ri), *Ephestia kuehniella* and *Cadra cautella* (*w*CauA, *w*CauB, *w*Kue, *w*Sca) all have WO prophages present, based upon screening for the WO minor caspsid protein gene (orf7) (Masui et al., 2000). Subsequently, every genome sequence of arthropod strains of *Wolbachia* has revealed numerous prophages (Wu et al. 2004; Klasson et al., 2008; Klasson et al., 2009). The genomes of purified bacteriophage particles from wCauA- and wCauB-*Wolabachia* infecting *E. kuehniella* (Fujii et al., 2004; Tanaka et al., 2009), and *w*VitA and *w*VitB-*Wolbachia* infecting *Nasonia vitripennis* (Kent et al., 2011) have been sequenced.

Although the bacteriophage WO is genetically isolated as a result of *Wolbachia*'s intracellular lifestyle, it is responsible for much of the genome diversification of *Wolbachia*. WO is capable of horizontal gene transfer in arthropod hosts coninfected with two different strains of *Wolbachia* (Kent et al., 2011). Currently, the role of WO prophages in the interaction between *Wolbachia* and insect hosts is poorly described, nor are the functions of proteins encoded by the prophage genome understood. Interestingly, expression of ankyrin motif-containing genes found in prophages is involved in feminization of isopods (Pichon et al., 2012). Also, lytic WO phages modify cytoplasmic incompatability levels in *N. vitripennis*. The ability of WO to lyse its host and reduce *Wolbachia* density decreases the ability of *Wolbachia* to induce cytoplasmic incompatability (Bordenstein et al., 2006). Lytic WO in *Wolbachia w*Vit can be induced by temperature stress (Bordenstein and Bordenstein, 2011). Using quantitative PCR to identify which of the threeWO phages in wRi was capable of replicating,

Biliske et al. (2011) were able to determine that only WORiC was active, generating extrachromosmal copies of phage genes.

The WO bacteriophage is a source of recombination in *Wolbachia* (Ishmael et al., 2009; Bordenstein and Werengreen, 2004). Horizontal transfer of phages between *Wolbachia* strains coinfecting a single host has been reported in the parasitoid *Nasonia vitripennis* (Kent et al., 2011) and the leaf beetle *Neochlamisus bebbianae* (Chafee et al., 2010) and acts as a mechanism for acquiring new genes through horizontal gene transfer. The transfer of potential effector molecules that have a function in the biology of *Wolbachia* may account for the phenotypic difference seen among *Wolbachia* strains.

The intracellular lifestyle limits the transfer of genes between *Wolbachia* strains; however there is the potential to transfer genes from other endosymbionts such as *Rikettsia* endosymbiont of the tick *Ixodes scapularis* to *Wolbachia* (Ishmael et al., 2009). The bacteriophage WO is a potential vector for horizontal gene transfer of *Wolbachia* genes to the eukaryotic host, as is observed in *Aedes aegypti* in which WO prophage genes are associated with the integrated *Wolbachia* genes (Klasson et al., 2009). WO may be the vector for other observed prokaryote-eukaryote horizontal gene transfer (Hotopp et al., 2007; Nikoh et al., 2009). The potential to use the WO bacteriophage for the transformation of *Wolbachia* is important for future research as currently no methods exist for genetically manipulating *Wolbachia* (Metcalf and Bordenstein, 2012).

This study compares the genomes between known active phages WOVitA1 and WOCauB2 found in *N. vitripennis* and *C. cautella* respectively and the

uncharacterized prophages of wMel in *D. melanogaster* and wRi in *D. simulan*s in order to examine conservation of shared genome structure. This comparison identifies regions of similarity between these phages and estimates the core set of genes required to generate virus particles. Since each sequenced WO prophage region has undergone numerous rearrangements, whole genome alignments were used to identify regions of collinearity. Comparative analysis of the genome architecture of these prophages identified the conserved genomic backbone in different WO strains. My results identify modules for head assembly and DNA packaging as well as tail morphogenesis present in wMel and wRi and conserved in all known active WO phages. These regions can be used to predict potentially active phages in future sequencing projects.

Materials and methods

Sequence analysis

Annotated genomes of *Wolbachia* strains wMel (GenBank:NC_002978) (Wu et al., 2004) and wRi (GenBank:NC_012416) (Klasson et al., 2009), and phage strains WOCauB2 (GenBank:AB478515) (Tanaka et al., 2009), and WOVitA (GenBank:HQ906662) (Kent et al., 2011) were retrieved (Benson et al., 2008). The phage regions WRi_005250-005970 in WORiB and WRi_006570-WRi_007250 in WORiC from the *w*Ri genome were used for whole phage genome alignments. The region WD0562-WD0646 from the *w*Mel genome was used for WOMelB genome alignments. Whole genome comparisons were performed using the Mauve plug-in v.2.2.0 (Darling et al., 2004) for Geneious

v5.4.4 (Drummond et al., 2011). The predicted amino acid sequences for the large terminase subunit and baseplate assembly gene W were used for phylogenetic analysis to determine the evolutionary history of different components of the core bacteriophage genome.

Proteins were aligned using the ClustalW multiple alignment algorithm implemented in Geneious v5.4.4. (Drummond et al., 2011). Model selection was performed using Prottest 2.4 (Abascal et al., 2005) with Akaike's information criterion (AIC) to select for an appropriate evolutionary model for each data set prior to analysis: terminase (JTT+I+Γ+F) and baseplate assembly protein W (JTT+Γ). Maximum likelihood analysis was conducted using PHYML with the JTT substitution model with gamma correction for terminase (estimated proportion of invariable sites, four substitution rate category) and baseplate assembly protein W (four substitution rate category) (Guindon and Gascuel, 2003). Bootstrap values were calculated from 1000 replicates replicates with NNI branch swapping

Results

Naming of WO phages followed the convention established by the published genome of *Wolbachia* from *D. melanogaster* which revealed two integrated prophages WO-A and WO-B relative to their postion to the origin of replication (Wu et al., 2004). The sequencing of *D. simulans* showed four integrated prophages. Based on genome similarity with WO from *w*Mel, these were labeled as WO-A, two WO-B regions, and, with sharing little similarity to WOMel

phages, the last was named WO-C. The sequencing of the phage genomes from purified virions and subsequent *Wolbachia* genomes led to the convention of listing the *Wolbachia* strain origin between the WO and letter/number classification, such that WOMelA and WORiA are from *D. melanogaster* and *D. simulans* respectively. Thus, *D. simulans* genome has three distinct prophage regions named WORiA, WORiB, and WORiC.

The genome of the active WORiC prophage from *w*Ri is predicted to be 77,261 bp and to contain 56 ORFs (WRi _006570 to WRi_007250) (Biliske et al. 2011) (Figure 3.1). The core genome containing a DNA packaging and head assembly module and a tail morphogenesis module is 24.2 kbp (WRi_006910 to WRi_007210). The 35% GC content of the phage genome is identical to the GC content of the *w*Ri genome indicating a long period of co-evolution between prophage and bacteria.

The WO genomes isolated from active virus particles that have been sequenced to date belong to *Wolbachia* of *C. cautella*, WOCauB2 and WOCauB3 (Tanaka et al., 2009; Kent et al., 2011). More recently, Kent et al. (2011) used microarrays to capture the sequences of WOVitA and WOVitB which are the active phages in *w*VitA and *w*VitB respectively, infecting *N. vitripennis*. In my study, genomes from these active phages were compared to WORi prophage genomes to identify conserved regions. Figure 3.2 shows the overall gene synteny between the WO phages. In this figure the heights of the coloured peaks represent the degree of nucleotide similarity between collinear genomes. Pairwise alignments were performed between WORiC and WOCauB2

(Figure 3.2 A A), WORiC and WOVitA1 (Figure 3.2 B), WORiC and WORiB (Figure 3.2 C) and WOMelB (Figure 3.2 D). Additionally, pairwise alignments between WORiA and WOMelA to WORiC and WORiB were also performed (Figure 3.3 A-D). The WOMelB sequence used for comparisons included the upstream adjacent pyocin region identified by Wu et al (2004). These comparisons revealed conserved regions of homologous sequence and identified rearrangements and inversions between the genomes. The genes encoding putative structural and packaging proteins are present in two adjacent and conserved regions in WORiC, WOVitA1 and WOCauB2. WORiA and WOMelA did not align with other WORi phage genomes (Figure 3.3 A-D).

Comparisons between WORiC and WOCauB2 reveal a single block of homologous sequences spanning the structural and packaging regions (Figure 3.2 A). There are three separate areas of dissimilarity between WORiC and WOCauB2. These include two transposable elements and an uncharacterized phage protein (WRi_007190).

Notable areas of dissimilarity between WOVitA1 and WORiC (white areas; Figure 3.2 B) include two transposable elements (WRi_006820) interrupting an ankyrin repeat protein gene (WRi_006810, WRi_p06840).The major region of dissimilarity between WOVitA1 and WORiC, adjacent to the structural module in WOVitA1, could be a result of horizontal gene transfer into WOVitA1 or gene loss in WORiC. These ORFs in WOVitA1 encode MutL and three transcriptional regulators (ADW80184.1, ADW80182.1 to ADW80179.1). Although WOVitA1

and WORiC share 36 homologs compared to 33 shared between WORiC and WOCauB2, WORiC is more similar to WOCauB2 (92.4%) than WOVitA1.

Genome alignments were also used to assign possible functions to previously annotated hypothetical ORFs. A hypothetical gene, (WRi_p07030), shares 74.7% pairwise identity to the virulence protein gene VrlC.1 of WOVitA1 and is pseudonized by the transposon insertion (WRi_007040). The annotated hypothetical protein (WRi _007070) is homologous to tail protein I from WOVitA1 (96%, 3e-143).

The WORiB genome shares only ORFs found within the packaging region (WRi_005460 to WRi_005610) with WORiC (figure 3.2 C). This packaging region is also conserved in WORiA with the addition of two upstream ORF's coding for ankyrin proteins (WRi_005440 to WRi_5610) (Figure 3.2 B). Between WORiA and WORiB (data not shown) the points of dissimilarity include two transposable elements (WRi_012560 and WRi_012520). One of these (WRi_012520) appears to have moved within the packaging region and shares 100% nucleotide similarity with the transposase adjacent to the packaging region of WORiB (WRi_005420). ORF's coding for a PQQ repeat containing enzyme (WRi_012580) and a hypothetical protein (WRi_012620) are present in WORiA but absent in WORiB. These differences also occur between WORiC and WORiA, as well as an additional transposable element with a frameshift mutation (WRi_p12500) and a lysozyme gene (WRi_012650) instead of the uncharacterized phage protein (WRi_007190) that is only found within WORiC.

The comparison of WOMelA to WORiC shows the conservation of the DNA packaging and head assembly region (WRi_007070 to WRi_007210) and similarities between ankyrin repeat containing genes. WOMelA shares a large region of homologous sequence with WORiB that includes aParB-methyltransferase gene (WRi_005640).

When the pyocin sequences, containing the viral structural genes, are included in the WOMelB genome and aligned with WORiC, the structural and packaging regions are conserved, but rearranged in WOMelB compared to WORiC (Figure 3.2 D).

The evolutionary relationships of the tail morphogenesis and head assembly module and the DNA packaging module were examined by phylogenetic analysis. Phylogenetic trees based on baseplate assembly protein W (tail morphogenesis) and the large terminase subunit (DNA packaging) showed different evolutionary relationships for related phages, with the exception of the WOMelB, WORiB1 and WORiB2 clade (Figure 3.4). WORiC shows the greatest phylogenetic relatedness to WOCauB2 and WOCauB3 for baseplate assembly protein W (Figure 3.4 A), which is indicated by the high degree of nucleotide similarity (97.8% pairwise identity) in the alignment (Figure 3.2 A). In contrast, the large terminase subunit of WORiC is most closely related to the *w*Mel and *w*Ri B-type phages (Figure 3.4 B).

Discussion

Using quantitative PCR to identify which of the four WO phages in the wRi genome was capable of replicating, Biliske et al. (2011) determined that only WORiC was active, generating extrachromosmal copies of phage genes. In this study, a comparative genomic approach was taken to identify the core genome conserved between WORiC and two previously characterized temperate bacteriophages WOVitA1 (from *Wolbachia* of *N. vitripennis*) and WOCauB2 (from *Wolbachia* of *C. cautella*), in order to identify putative essential regions required for phage generation. The genomes of WORiC, WOVitA1, and WOCauB2 show considerable sequence homology which supports the view that WORiC is the active form of phage in *w*Ri. The two conserved modules shared by WORiC and the temperate phages WOCauB2 and WOVitA1 include the DNA packaging and head assembly region and the tail morphogenesis region (Figure 3.2A-B). In contrast, the WORiB genome and the WOMelB genome lacking the upstream pyocin region share few homologous sequences with WORiC. Genes with sequence homology in WORiB, WOMelB, and WORiC belong to the DNA packaging and head assembly region. However, the core tail structural region of WORiC aligns with WOMelB once the pyocin region is included in the analysis. WORiB lacks the pyocin-like region and is therefore deficient in most tail morphogenesis genes.

The chimeric nature of WO phages was initially identified by Masui et al. (2001), who described the large terminase subunit, portal protein and minor capsid protein of the DNA packaging region in WOKue as lambda-like, and the

baseplate assembly proteins of the tail structural region as P2-like. This hybridization of lambda and P2 sequences is not exclusive to WO phages, since chimeric phages have been described in other systems; for example *Xylella fastidiosa* phages XfP1 and XfP2 are also lambda/P2 chimeras (Canchaya et al., 2003). Due to recombination and genetic mosaicism, different parts of a bacteriophage genome can have different evolutionary histories (Lawrence et al., 2002). In the chimeric WO phages (Figure 3.4), the large terminase subunit sequence from the DNA packaging and head assembly regions shows a different phylogenetic relationship than the baseplate assembly protein W sequence from the tail morphogenesis regions. This modular nature of WO phages has been described previously (Kent and Bordenstein, 2010). I have shown that the most conserved region shared by all sequenced WO bacteriophages is the DNA packaging region.

The genome encoding the DNA packaging and head assembly module includes ORFs that putatively code for a portal protein, a minor capsid protein and the large subunit of the terminase protein (Figure 3.1). This large terminase subunit contains a DNA-dependent ATPase domain and site-specific nuclease domain which are both involved in DNA translocation during packaging. In double-stranded DNA phages, terminases are generally accompanied by a small subunit involved in DNA binding (Black, 1989; Rao and Feiss, 2008). However, no homolog of this small subunit has been identified in any WO genome. The portal protein of tailed bacteriophages forms a complex with the terminase proteins which translocates phage DNA into the prohead during phage replication

(Rao and Feiss, 2008). The conservation of these packaging genes suggests that DNA packaging in WO phages is driven by an ATP-dependent DNA translocation motor similar to other tailed bacteriophages.

Similarly, the organization of the tail morphogenesis module is conserved among WOVitA, WOCauB, and WORiC. Genes involved in tail assembly include the tail proteins, tail tape measure protein, the tail sheath protein, the contractile tail tube protein and baseplate assembly proteins J,W, and V. Tail morphogenesis in the subfamily Myoviridae, which have long contractile tails, is the most complex of all tailed bacteriophages. In the Myoviridae, T4, P2 or Mu, baseplate assembly occurs first and is required for sheath and tail polymerization. It is from the baseplate that the tube polymerizes to a length determined by the tail-tape measure protein and this is followed by the tail sheath which extends the length of the tail (Leiman et al., 2010).

The presence of the tail sheath gene in active WO genomes suggests that, with respect to tail structure and assembly, these phages are more similar to Myoviridae than to the subfamily Siphoviridae, which includes lambda and lacks contractile tails. The phage tail mediates genome delivery into host cells, and is required for the generation of infectious phages. The absence of this region in the WORiB genome may contribute to the inability of WORiB to form infectious particles.

Unlike WORiC, in which the packaging region is located adjacent to the structural proteins, in WOMelB the structural proteins are divided in the genome and separated from the packaging region by approximately 18kbp (Iturbe-

Ormaetxe et al., 2005). One region of structural genes found in WOMelB was initially characterized as a pyocin-like region. Therefore, active phage generation in *D. melanogaster w*Mel could result from the coordinated replication of both packaging and structural regions. Despite much previous interest in *Wolbachia*'s ankyrin containing genes (Iturbe-Ormaetxe et al., 2005; Duron et al., 2007), and the suggestion that they may influence phage function, the ORFs encoding ankyrin-containing motifs are outside the core conserved regions of WORiC, WOVitA1 and WOCauB3. The role of ankyrin coding genes in the WO-*Wolbachia*-host relationship remains elusive (Walker et al., 2007, Yamada et al., 2010).

My results suggest that *Wolbachia* phages WORiC and known active phages WOCauB and WOVitA1 represent a conserved class of *Wolbachia* phages. Interest in the conserved genetic modules of the lambda-like DNA packaging and head assembly genes and P2-like tail morphogenesis genes led to the investigation of the relatedness of the *Wolbachia* phages. Phylogenetic analysis shows similarity between WORiC and WO-B's found in *w*Mel and *w*Ri (based on large terminase subunit phylogeny) and similarity between WORiC and WOCauB2 and WOCauB3 (based on the baseplate assembly protein W phylogeny). These divergent topologies are indicative of the horizontal transfer events occurring between phage genomes. Similarity of genomes of active WO phages may be due to the fact that they have a common, recent origin, or because active WO phages are operating within a limited framework of endosymbiotic bacteria, where opportunities for incorporating novel gene sequences by recombination are

limited. Given the present level of knowledge of active WO bacteriophages, I cannot distinguish between these and other possible evolutionary scenarios.

Figures

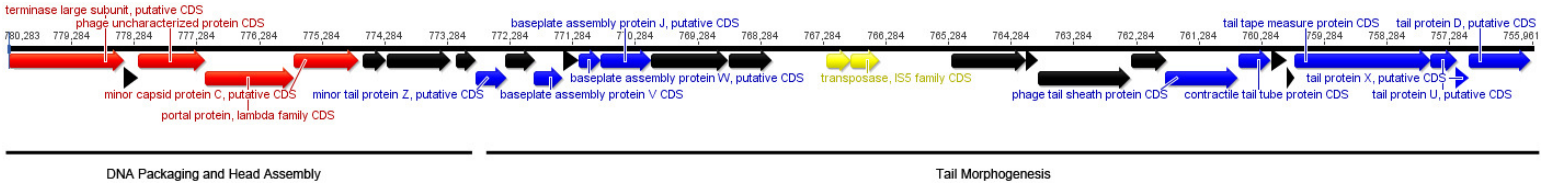


Figure 3.1 Protein map of the conserved core DNA packaging and head assembly module and tail morphogenesis module of WORiC found in wRi (Klasson et al., 2009) generated using Geneious v5.4.4 (REF).

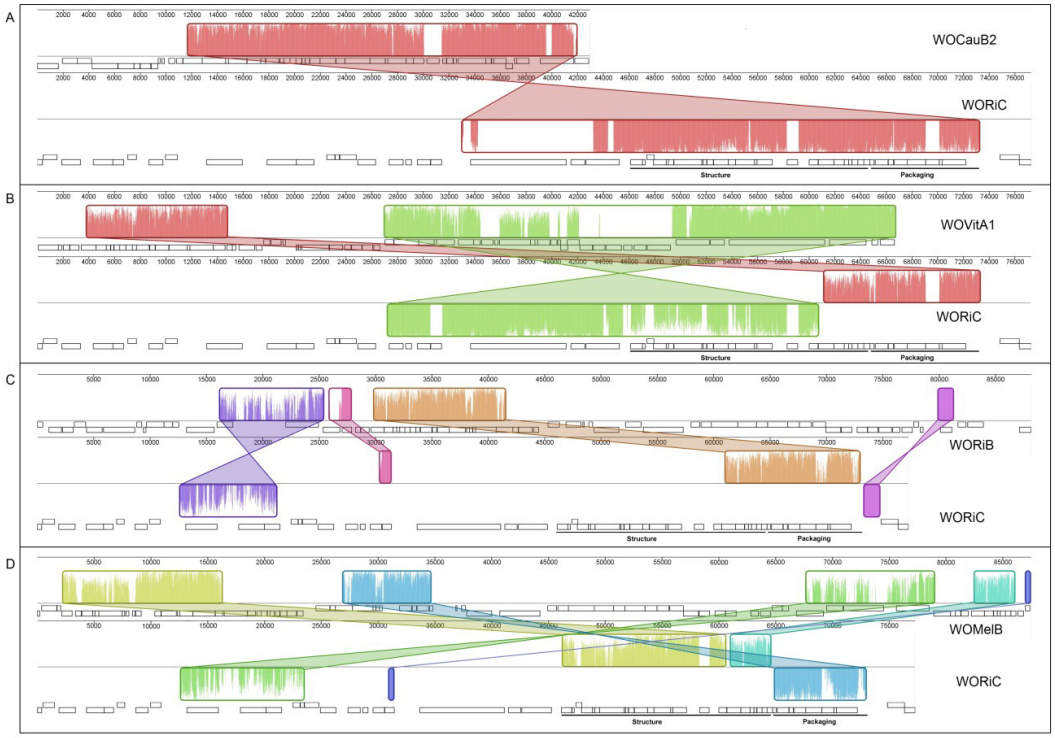


Figure 3.2: Whole genome comparisons between WORiC, WOCauB2, WOVitA1, WOMelB, and WORiB. Genomes of WORiC to A) WOCauB2 B) WOVitA1 C) WOMelB and D) WORiB are compared. Degree of sequence similarity is represented by the peak height within each block. Areas of white within blocks indicate dissimilarity including gene insertions or deletions (see text). Each coloured block represents a conserved region of homologous segments between genomes. The placement of a block below the center axis indicates inverted regions.

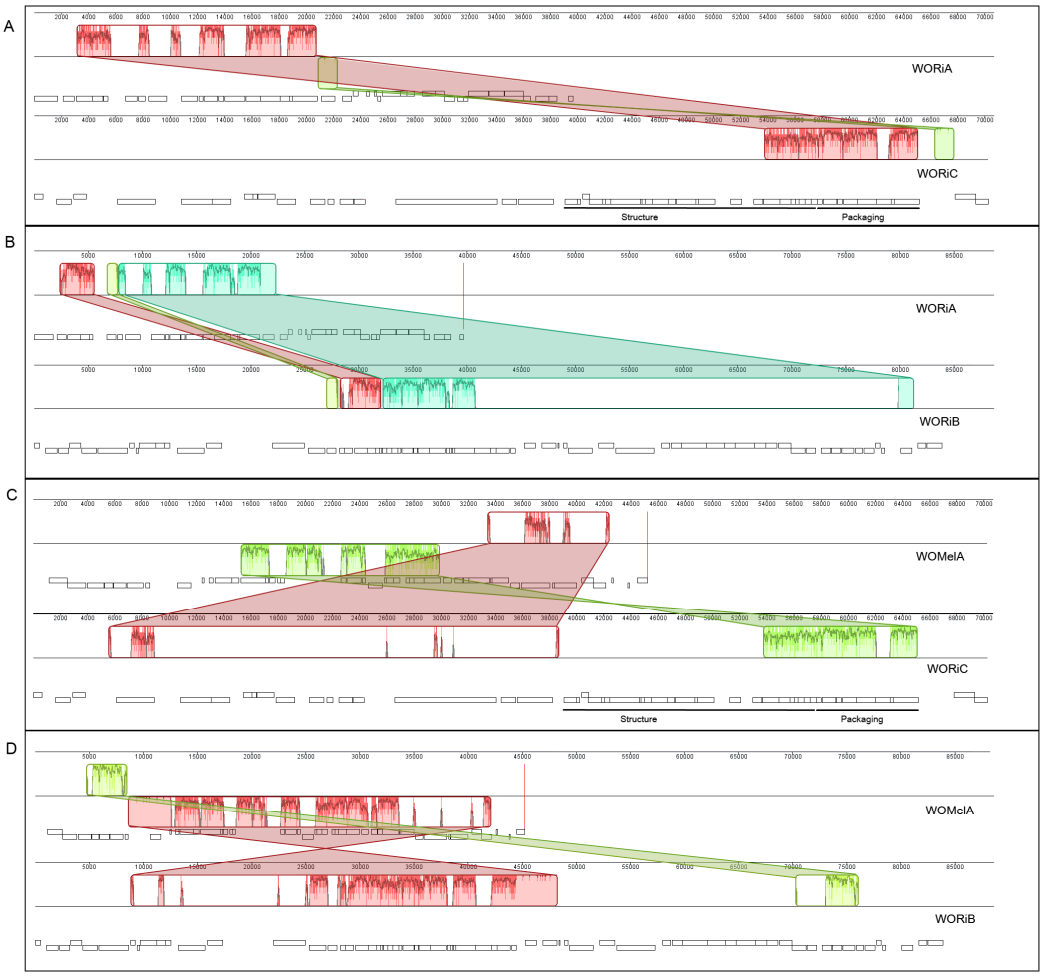


Figure 3.3: Whole genome comparisons between WORiA, WOMelA, WORiC, and WORiB. Genomes of WORiA compared to A) WORiC B) WORiB. Genomes of WOMelA to C) WORiC and D) WORiB are compared. Areas of white within blocks indicate dissimilarity including gene insertions or deletions. Each coloured block represents a conserved region of homologous segments between genomes. The placement of a block below the center axis indicates inverted regions.

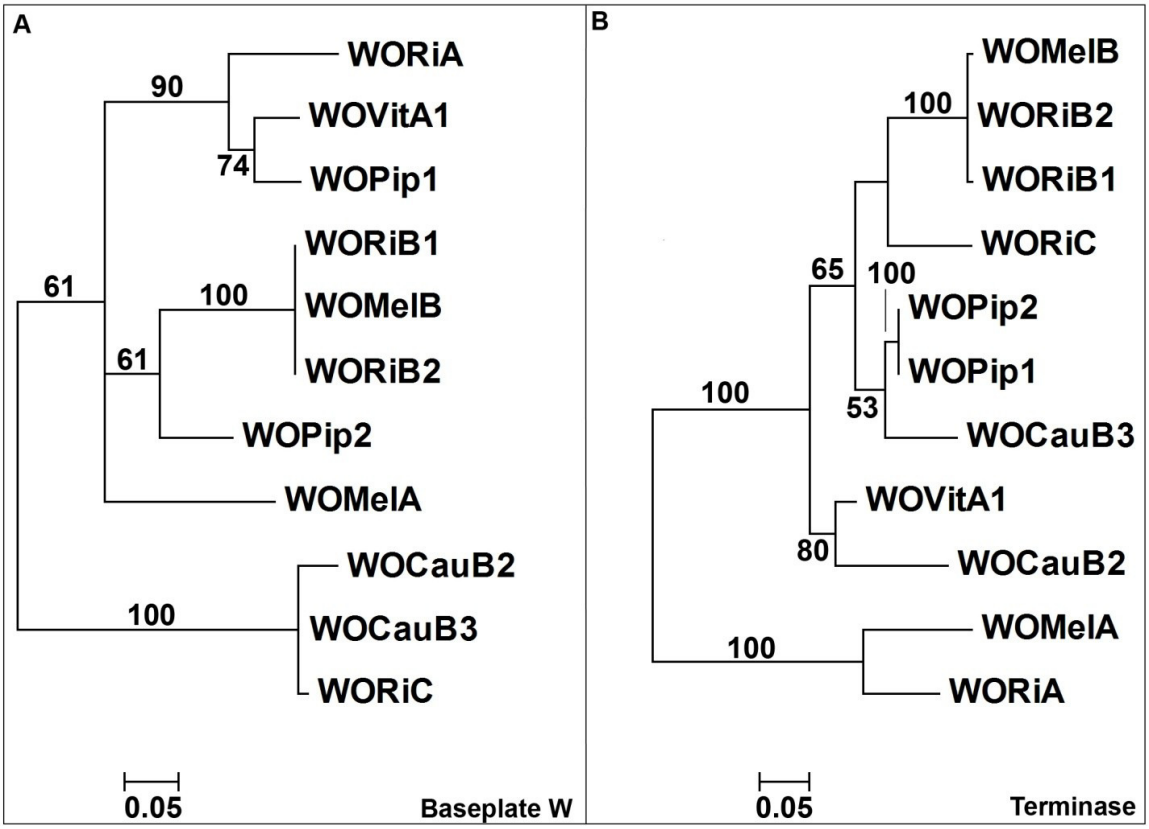


Figure 3.4: **Phylogeny of terminase and baseplate assembly protein W amino acid sequences**. Maximum-likelihood phylogeny based on translated amino-acid sequences of A) baseplate assembly gene W (tail morphogenesis module) and B) large terminase subunit gene (DNA packaging and head assembly module) of *Wolbachia* WO phages from published genomes. Trees of highest likelihood are shown. Bootstrap values for each node are based on 1000 resamplings.

References

Abascal F, Zardoya R, Posada D. 2005. ProtTest: Selection of best-fit models of protein evolution. Bioinformatics, 21:2104-2105.

Ackermann, H.-W., DuBow, M.S. 1987. Viruses of Prokaryotes. Vol. II. General Properties of Bacteriophages, pp. 1–54 and 171–218. CRC Press, Boca Raton, FL.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. 2008. GenBank. Nucleic Acids Research, 36(Database issue):D25-30.

Biliske JA, Batista PD, Grant CL, Harris HL. 2011. The bacteriophage WORiC is the active phage element in wRi of *Drosophila simulans* and represents a conserved class of WO phages. BMC Microbiology, 11:251.

Black L. 1989. DNA packaging in dsDNA bacteriophages. Annual Review of Microbiology, 43:267-292.

Brüssow H, Canchaya C, Hardt WD. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiology and Molecular Biology Reviews, 68(3):560-602.

Brüssow H, Hendrix RW. 2002. Phage genomics: small is beautiful. Cell, 108:13-16.

Bordenstein SR, Bordenstein SR. 2011. Temperature affects the tripartite interactions between bacteriophage WO, *Wolbachia*, and cytoplasmic incompatibility. PLoS One, 6(12):e29106.

Bordenstein SR, Marshall ML, Fry AJ, Kim U, Wernegreen JJ. 2006. The tripartite associations between bacteriophage, *Wolbachia*, and arthropods. PLoS Pathogens, 2(5):e43

Bordenstein SR, Wernegreen JJ. 2004. Bacteriophage flux in endosymbionts (*Wolbachia*): Infection frequency, lateral transfer, and recombination rates. Molecular Biology and Evolution, 21:1981–1991.

Botstein D. 1980. A theory of modular evolution for bacteriophages. Annals of the New York Academy of Sciences, 354:484-90.

Canchaya C, Proux C, Fournous G, Bruttin A, Brüssow H. 2003. Prophage Genomics. Microbiology and Molecular Biology Reviews, 67(2):238-276.

Casjens S. 2003. Prophages in bacterial genomics: what have we learned so far? Microbiology, 249:277–300.

Chafee ME, Funk DJ, Harrison RG, Bordenstein SR. 2010. Lateral phage transfer in obligate intracellular bacteria (*Wolbachia*): verification from natural populations. Molecular Biology and Evolution, 27(3):501-5.

Chibani-Chennoufi S, Bruttin A, Dillmann ML, Brüssow H. 2004. Phage-host interaction: an ecological perspective. Journal of Bacteriology, 186(12):3677-86.

Darling AC, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Research, 14(7):1394-403.

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A. 2011. Geneious. Version 5.4. http://www.geneious.com.

Dunning Hotopp JC, Clark ME, Oliveira DC, Foster JM, Fischer P, Muñoz Torres MC, Giebel JD, Kumar N, Ishmael N, Wang S, Ingram J, Nene RV, Shepard J, Tomkins J, Richards S, Spiro DJ, Ghedin E, Slatko BE, Tettelin H, Werren JH. 2007. Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. Science, 317(5845):1753-6

Duron O, Boureux A, Echaubard P, Berthomieu A, Berticat C, Fort P, Weill M. 2007. Variability and Expression of ankyrin domain genes in *Wolbachia* variants infecting the mosquito *Culex pipiens*. Journal of Bacteriology, 180(12):4442-4448.

Fujii Y, Kubo T, Ishikawa H, Sasaki T. 2004. Isolation and characterization of the bacteriophage WO from *Wolbachia*, an arthropod endosymbiont. Biochemical and Biophysical Research Communications. 317, 1183–1188.

Glazko G, Makarenkov V, Liu J, Mushegian A. 2007. Evolutionary history of bacteriophages with double-stranded DNA genomes. Biology Direct, 2:36.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Sytematic Biology, 52:696-704.

Hatfull GF. 2008. Bacteriophage genomics. Current Opinion in Microbiology, 11(5):447-53.

Hayashi T, Makino K, Ohnishi M, Kurokawa K, Ishii K, Yokoyama K, Han CG, Ohtsubo E, Nakayama K, Murata T, Tanaka M, Tobe T, Iida T, Takami H, Honda T, Sasakawa C, Ogasawara N, Yasunaga T, Kuhara S, Shiba T, Hattori M, Shinagawa H. 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. DNA Research, 8(1):11-22.

Iguchi A, Iyoda S, Terajima J, Watanabe H, Osawa R. 2006. Spontaneous recombination between homologous prophage regions causes large-scale inversions within the *Escherichia coli* O157:H7 chromosome. Gene, 372:199-207.

Ishmael N, Dunning Hotopp JC, Ioannidis P, Biber S, Sakamoto J, Siozios S, Nene V, Werren J, Bourtzis K, Bordenstein SR, Tettelin H. 2009. Extensive genomic diversity of closely related *Wolbachia* strains. Microbiology, 155(Pt 7):2211-22.

Iturbe-Ormaetxe I, Burke GR, Riegler M, O'Neill SL. 2005. Distribution, expression, and motif variability of ankyrin domain genes in *Wolbachia pipientis*. Journal of Bacteriology, 187(15):5136-5145.

Kent B, Bordenstein SR. 2010. Phage WO of *Wolbachia*: lambda of the endosymbiont world. Trends in Microbiology, 18(4):173-181

Kent B, Salichos L, Gibbons J, Rokas A, Newton I, Clark M, Bordenstein SR. 2011. Complete bacteriophage transfer in a bacterial endosymbiont (*Wolbachia*) determined by targeted genome capture. Genome Biology and Evolution, 3:209-218.

King AMQ, Lefkowitz E, Adams MJ, Carstens EB. 2012. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses (ICTV). Elsevier Academic, San Diego, CA

Klasson L, Walker T, Sebaihia M, Sanders MJ, Quail MA, Lord A, Sanders S, Earl J, O'Neill SL, Thomson N, Sinkins SP, Parkhill J. 2008. Genome evolution of *Wolbachia* strain wPip from the *Culex pipiens* group. Molecular Biology and Evolution, 25(9):1877-1887.

Klasson L, Westberg J, Sapountzis P, Näslund K, Lutnaes Y, Darby AC, Veneti Z, Chen L, Braig HR, Garrett R, Bourtzis K, Andersson SG. 2009. The mosaic genome structure of the *Wolbachia* wRi strain infecting *Drosophila simulans*. Proceedings of the National Academy of Sciences USA, 106(14):5725-30

Lawrence JG, Hatfull GF, Hendrix RW. 2002. Imbroglios of viral taxonomy: Genetic exchange and failings of phenetic approaches. Journal of Bacteriology, 184(17):4891-4895.

Leiman PG, Arisaka F, van Raaij MJ, Kostyuchenko VA, Aksyuk AA, Kanamaru S, Rossmann MG. 2010. Morphogenesis of the T4 tail and tail fibers. Virology Journal, 7:355.

Maniloff J, Ackermann HW. 1998. Taxonomy of bacterial viruses: establishment of tailed virus genera and the order Caudovirales. Archives of Virology, 143(10):2051-63.

Masui S, Sasaki T, Ishikawa H. 2000. Genes for the type IV secretion system in an intracellular symbiont, *Wolbachia*, a causative agent of various sexual alterations in arthropods. Journal of Bacteriology, 182(22):6529-6531.

Masui S, Kuroiwa H, Sasaki T, Inui M, Kuroiwa T, Ishikawa H. 2001. Bacteriophage WO and virus-like particles in *Wolbachia*, an endosymbiont of

arthropods. Biochemical and Biophysical Research Communications, 283(5):1099-1104.

Metcalf JA, Bordenstein SR. 2012. The complexity of virus systems: the case of endosymbionts. Current Opinion in Microbiology, 15(4):546-52.

Nikoh N, Nakabachi A. 2009.Aphids acquired symbiotic genes via lateral gene transfer. BMC Biology, 7:12.

Ohnishi M, Terajima J, Kurokawa K, Nakayama K, Murata T, Tamura K, Ogura Y, Watanabe H, Hayashi T. 2002. Genomic diversity of enterohemorrhagic *Escherichia coli* O157 revealed by whole genome PCR scanning. Proceedings of the National Academy of Sciences USA, 99(26):17043-8.

Pichon S, Bouchon D, Liu C, Chen L, Garrett RA, Grève P. 2012. The expression of one ankyrin pk2 allele of the WO prophage is correlated with the *Wolbachia* feminizing effect in isopods. BMC Microbiology, 12:55.

Rao VB, Feiss M. 2008. The bacteriophage DNA packaging motor. Annual Review of Genetics, 42:647-681.

Tanaka K, Furukawa S, Nikoh N, Sasaki T, Fukatsu T. 2009. Complete WO phage sequences revealed their dynamic evolutionary trajectories and putative functional elements required for integration into *Wolbachia* genome. Applied and Environmental Microbiology, 75(17):5676-5686.

Walker T, Klasson L, Sebaihia M, Sanders MJ, Thomson N, Parkhill J, Sinkins SP. 2007. Ankyrin repeat domain-encoding genes in the wPip strain of *Wolbachia* from the *Culex pipiens* group. BMC Biology, 5:39.

Weinbauer MG. 2004. Ecology of prokaryotic viruses. FEMS Microbiology Reviews, 28(2):127-81.

Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadinejad N, Wiegand C, Madupu R, Beanan MJ, Brinkac LM, Daugherty SC, Durkin AS, Kolonay JF, Nelson WC, Mohamoud Y, Lee P, Berry K, Young MB, Utterback T, Weidman J, Nierman WC, Paulsen IT, Nelson

KE, Tettelin H, O'Neill SL, Eisen JA. 2004. Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: A streamlined genome overrun by mobile genetic elements. PLoS Biology, 2(3):0327.

Yamada R, Iturbe-Ormaetxe I, Brownlie JC, O'Neill SL. 2010. Functional test of the influence of *Wolbachia* genes on cytoplasmic incompatibility expression in *Drosophila melanogaster*. Insect Molecular Biology, 20(1):75-85.

Chapter 4

Molecular and phylogenetic characterization of the WO-encoded ParB-Methyltransferase in *Wolbachia*

Introduction

Numerous epigenetic systems control gene expression in organisms. According to Cassadesus and Low (2006), epigenetic gene regulation occurs when gene expression is affected without any modification to the DNA sequence. The simple addition of a methyl group to amino acid residues in histones or nucleotides in DNA alters the state of those molecules and in turn changes gene expression. In the case of DNA methylation, the epigenetic modification is the presence or absence of a methylated base which can prevent or enhance transcription factors from actively binding to their target site. The presence of a methyl group on a base adds additional information to the DNA molecule, without changing the gene sequence.

DNA methylation was first discovered by Hotchkss (1948) in DNA from calf thymus and subsequently has been described in virtually all living systems (Cheng, 1995). Methylation of DNA is carried out by enzymes known as DNA methyltransferases (MTases). All known MTases methylate DNA by transferring a methyl group from the cofactor S-adenosyl-$_{L}$-methionine (AdoMet) to a target base, either adenine or cytosine (Bujnicki and Radlinska, 1999). These bases are known to be methylated by MTases within or near a recognition site of 4-7 base pairs. All known MTases share structurally similar domains and motifs. In spite of

The work presented here (including data generated and data analysis) was carried out by the author. A portion of this data has been published in Saridaki et al., 2011. PLoS One, 6(5):e19708

these structural similarities MTases are classified according to how they interact with the target bases, the catalytic mechanisms used, and their evolutionary history.

On the basis of catalytic mechanisms of the addition of AdoMet to nucleotide, DNA MTases are grouped into two classes (Jeltsch, 2002). One class forms C5-methylcystosine (5mC) and a second class forms N6-methyladenine (N6mA) and N4-methylcytosine (N4mC) (Cheng, 1995). In the formation of 5mC, a covalent intermediate is formed between a Cys residue in the MTase and the C6 atom of cytosine. This covalent bond leads to the addition of a methyl group from AdoMet (Cheng, 1995). It is only after this step that the enzyme is released (Jeltsch, 2002). Unlike the C-MTases, the N4- and N6-MTases do not form this covalent intermediate. In both classes, enzyme binding to a target site results in a conformational change in both the DNA and the protein, where the nucleotide that will be methylated is flipped out into the active site of the enzyme in order to be methylated (Jeltsch, 2002; Cheng 1995).

The structure of MTases consists of two conserved domains, the large and small domain. The small domain is the target recognition domain (TRD), which is responsible for recognition of the target site (Jeltsch, 2002). The large domain, which differs greatly between cytosine-MTases and adenine-MTases, is composed of up to 10 conserved motifs (Jeltsch, 2002). The large domain of N-MTases is made up of nine conserved motifs (Malone, 1995). The two most conserved motifs are motif I, which is the region that binds to methionine in AdoMet and motif IV, also called the DPPY-motif, which is involved in catalysis of methyl

transfer between AdoMet and the target base. Based on the order of these nine motifs and the position of TRD, adenine-MTases can be further subdivided into 3 groups: the α-, β-, and γ-group. The order of the α-group is motif I-TRD-motif IV, the order of the β-group is motif IV-TRD-motif I, and the order of the γ-group is motif I-motif IV-TRD (Malone et al., 1995). Adenine-MTases occur in all three groups (Jeltsch, 2002).

The phylogenetic relationship of N4-MTases and N6-MTases shows that these two families of MTases have diverged from a common ancestor (Bujnicki and Radlinska, 1999). It has been suggested that the origin of N4-MTases was from a β-group N6-MTase, which would mean that the α-group of N4 and N6-MTases arose independently of one another (Bujnicki and Radlinska, 1999). Phylogenetic analyses of various N-MTase genes by Bujnicki and Radlinska (1999) shows that these genes underwent frequent horizontal gene transfer between bacteria and Archaea. A lack of congruence between organismal and gene topologies suggests HGT, but discrepancies may also be the result of other biological processes, such as recombination or orthologous replacement (Paptsova and Gogarten, 2007).

5mC methylation is found in most eukaryotes, whereas N6mA and N4mC are primarily or exclusively found in prokaryotes. In prokaryotes MTases either belong to a restriction-modification system or are considered orphan MTases. The biological role of these MTases in prokaryotes is diverse, ranging from viral defense using the R-M (Restriction-Modification) system to cell-cycle regulation and DNA repair (Jeltsch, 2002). In the R-M system there is a restriction endonuclease and a cognate DNA MTase (Wion and Casadesús, 2006). A

restriction endonuclease cleaves a target DNA sequence unless that site has been methylated by a related methyltransferase (Casadesús and Low, 2006). Since the DNA MTase ensures that genomic and plasmid DNA is completely methylated, any foreign DNA, such as that from a bacteriophage, is not methylated and is subsequently cleaved and destroyed (Casadesús and Low, 2006). Many of the R-M systems are regulated by their associated MTases, which suggests a possible evolutionary link between MTases in the R-M system and the orphan MTases which are commonly involved in epigenetic gene regulation.

The roles of orphan MTases in controlling cell cycle and DNA repair are best described in the *Escherichia coli* dam (for *d*NA *a*denine *m*ethyltransferase) system and similar systems in other gammaproteobacteria, and the cell-cycle regulated DNA MTase (CcrM) family found in alphaproteobacteria (Casadesús and Low, 2006; Jeltsch, 2002). Dam is an α-group MTase which methylates the N6 atom of an adenine residue within a 5'-GATC-3' sequence. In *E. coli*, almost all the GATC sequences are methylated. The role of the Dam enzyme was determined by Heusipp et al. (2007) using Dam-overproducing and Dam mutant strains. These roles include regulating gene expression, DNA replication, and DNA repair (Wion and Casadesús, 2006). In DNA mismatch repair, removal of the region with the mismatch and re-synthesis of a new strand occurs near methylated sites (Løbner-Olesen et al., 2005). The sequestering protein SeqA binds to unmethylated 'GATC' sites, preventing DNA replication as well as chromosome segregation (Løbner-Olesen et al., 2005). The Dam enzyme controls DNA-protein interaction, whereby specific regulatory proteins will only bind to

hemimethylated (one methylated and one unmethylated DNA strand) or fully methylated (both strands are methylated) DNA, while other proteins bind to non-methylated DNA and block Dam methylation (Wion and Casadesús, 2006).

The β-group MTase known as CcrM was first described in the alphaproteobacterium *Caulobacter crescentus*. In this organism CcrM is essential for cell-cycle regulation (Wion and Casadesús, 2006). The CcrM protein methylates the newly synthesized strand of the bacterial chromosome after replication, which allows for the next cycle of replication by activating the transcriptional regulator *dnaA*. CcrM is only expressed and active during replication before being quickly degraded. Its activity is controlled by CtrA, which like SeqA, prevents DNA replication by binding to the origin of replication. Transcription of CcrM is activated by CtrA which leads to the accumulation of CcrM in the cell, and subsequently methylation of the newly synthesized strand of DNA, initiating another round of replication (Collier et al., 2007).

It is interesting that novel forms of MTases exist in some bacteria. An example is the β-group MTases with an N-terminal ParB-like nuclease domain (ParB-MTases) that appear to have resulted from a gene fusion event. This fusion gene has been described in six bacterial phyla; 47.5% are in the order alphaproteobacteria. ParB nucleases are part of the *parCBA* operon found in plasmids RK2 and RP4 (Johnson et al. 1999). The three cotranslated proteins of this operon include a recombinase (ParA), a calcium dependent nuclease (ParB) and ParC which has an unknown function. These proteins maintain low copy number plasmids within a growing bacterial population (Sobecky et al., 1996).

ParB is a monomer that has sequence homology to extracellular nucleases of *Staphylococcus aureus* (Johnson et al., 1999).

The function of the ParB-Mtase enzymes in prokayotes is presently under investigation. This gene is present along with CcrM in a number of bacterial genomes which suggests that unlike CcrM, ParB-Mtases have a nonessential role in cell viability. However, the presence of this gene in endosymbionts, including *Wolbachia*, which contain no other DNA MTases, suggests a role for this protein in the symbiotic interaction between the microbe and the host. The mechanism by which *Wolbachia* cell division is regulated within host cells has not been identified and the ParB-MTase may be involved in integrating replication of the bacterial cell with that of its host cells. Alternatively, it may be involved in epigenetically altering host DNA. In an earlier proteomic study, it was the only *Wolbachia* protein found in the egg cytoplasm of infected *Drosophila* embryos 15 min post fertilization (H. Braig and H. Harris, *personal communication*) suggesting that *Wolbachia* alters its host's DNA through methylation in the earliest stages of development. This is precisely the stage where rescue of modified sperm from infected males takes place, although the factors responsible for rescue remain to be identified.

The sequencing of the *Wolbachia* genomes infecting *Culex pipientis*, *C. quinquefasciatus*, *D. melanogaster* and *D. simulans* revealed the existence of the ParB-MTase within certain prophage regions. However, there has been no demonstration of adenine methylation of *Wolbachia* DNA or the DNA of infected hosts to date.

The purpose of this study is threefold: (i) to determine the phylogenetic relationships of the ParB-MTase genes present in bacteria, (ii) to analyze the gene structure of ParB-MTases and their genomic synteny, and (iii) to examine the extent of horizontal transferr of ParB-MTases between bacterial hosts.

My study examined the evolutionary history of the family of ParB-MTases in alphaproteobacteria. I focused on the incongruence of species (based on 16S ribosomal RNA (Williams et al., 2007)) and gene phylogenies to evaluate the extent of horizontal transfer. This study also investigated the conserved synteny of the genomic region containing ParB-MTases to identify possible functional role of this protein. In addition I attempted to express the recombinant protein in *E.coli* and determine DNA adenine MTase activity as well as nuclease activity of the ParB domain, although this part of the study is still ongoing.

Materials and Methods

Sequence Alignment and Model Selection

For *Wolbachia* ParB-MTases found in *Drosophila*, nucleotide and amino acid sequences (Saridaki et al., 2011) were aligned using the ClustalW Multiple Alignment algorithm implemented in Geneious v.5.3.3. (Drummond et al., 2010; Hall, 1999). The appropriate evolutionary model JTT+ Γ was selected by the Akaike Information Criterion (AIC) using Prottest v.2.4 (Abascal et al., 2005). Models of substitution for nucleotide alignments were selected using AIC in jModeltest v.0.1.1. (Darriba et al., 2012). The appropriate evolutionary model was TPM1uf+I+Γ for the MTase genes.

For the evaluation of the evolutionary history of ParB-MTases in prokaryotes from annotated genomes (Table 4.2), amino acid sequences were retrieved from the PIR protein database (Wu et al., 2002) and aligned using the ClustalW Multiple Alignment algorithm implemented in Geneious v5.4.4 (Drummond et al., 2011; Hall, 1999). The evolutionary model WAG+I+G+F appropriate for the *Wolbachia* ParB-MTase genes was selected by the Akaike Information Criterion (AIC) using Prottest v.2.4 (Abascal et al., 2005).

Phylogenetic analysis

For *Wolbachia* ParB-MTases found in *Drosophila*, the evolutionary history was inferred by the maximum likelihood (ML) criterion conducted for amino acid data using PHYML with the JTT substitution model (four substitution rate category) (Guindon and Gascuel, 2003). Bootstrap values were calculated from 100 replicates replicates with NNI branch swapping. ML trees generated were midpoint rooted.

The evolutionary history for prokaryotic ParB-MTases was determined using ML method using MEGA v5.2.2 (gamma distribution of rates at variable sites: alpha shape parameter = 1.5139; rate categories= 5). (Tamura et al., 2011). All positions containing gaps and missing data were eliminated. Bootstrap values were calculated from 100 replicates replicates with NNI branch swapping. Bootstrap values were added to the midpoint rooted tree with the highest log likelihood (-16207.4413).

To examine the evolutionary relationship between the terminase, portal protein and ParB-MTases, ML analysis was used using PHYML with the GTR substitution model (estimated proportion of invariable sites, and estimated gamma distribution) and NNI branch swapping. Trees of highest log likelihood scores are shown: terminase (-24759.77392), portal protein (-19676.86249) ParB-MTase (-19280.97995).

Evaluating Phylogenetic Congruence

In order to determine the occurrence of horizontal gene transfer of ParB-MTase between different bacterial taxa, congruence tests were applied to the organismal (16S) nucleotide data and the ParB-MTase gene of 10 species of alphaproteobacteria using the approximately unbiased (AU) test, and the Shimodaira-Hasegawa (SH) Test employed in CONSEL (Shimodaira-Hasegawa, 2001; Shimodoria and Hasegawa, 1999). For these tests optimal ML trees generated using the above models selected by the heuristic search method with 100 random sequence additions.

In order to test whether or not horizontal gene transfer occurred between different taxa, four different constraints were selected between the tree topologies. In each tree two different branches with high bootstrap support were selected and made monophyletic (constrained). For the 16S data set, two subsequent ML analyses were performed for constraints for the two highly supported branches in the ParB-MTase tree and for the ParB-MTases data set. I performed two additional ML analyses for constraints for the two highly supported branches in

the organismal phylogeny. If both the organismal and ParB-MTase trees were equally good at explaining the data (null hypothesis) which would mean that it shared and evolutionary history with the genome then I could reject the other alternative tree topologies suggesting that horizontal transfer did not occur.

Wolbachia ParB-MTase cloning and expression

Genome visualization, primer design, and in silico gateway cloning were done in Geneious v5.4.4. (Drummond et al., 2011). Primers were designed based on the Gateway cloning guidelines to amplify either the ParB or MTase domains based on sequence date (WRi_005640) (Table 4.2, Figure 4.1). In order to determine the result of the Gateway cloning an *in silico* experiment was run using retrieved plasmid sequences for both the donor vector (pDONR221) and destination vector (pDEST17). Simulation of the BP and LR reactions (Figure 4.2) was carried out in Genious v.5.4.4. (Drummond et al., 2011) in order to determine whether the destination vector would contain the in frame ParB or MTase domains for expression.

I used two rounds of PCR to amplify the attb-TEV-Gene-attb PCR product using five primers (Table 4.2). The first round of PCR amplification was designed to introduce a TEV protease site at the 5' end of the PCR product with attB sites flanking either the ParB or the MTase domain only. The second PCR was done to introduce the 5'-attb site to the TEV-Gene-attb. Each PCR reaction was done in a 100µl reaction mixture containing 1ul of template DNA, 10µl 10X reaction buffer, 8µl dNTP mixture (2.5mM each), 2.5µl 20mM of appropriate forward and reverse

primers, and 1µl of Taq DNA polymerase (Qiagen). The cycle parameters consisted of a 5 min initial denaturation step at 94˚C, followed by 30 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 1 min. Each PCR ended with a final 72˚C annealing step for 7 min. The first PCR reaction was set up to amplify a TEV-Gene-attb product using primers N1/N3 and C/C2. A subsequent PCR reaction was done using primers N2 and C/C2, using the previously purified PCR product as a template to get the final attb-TEV-Gene-attb required for cloning. Two reactions were carried out simultaneously. The final PCR product was cleaned up using a PCR purification kit (Qiagen) and the concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Each attB PCR product was diluted to a final concentration of 50 fmol prior to the BP reaction.

Construction of clone banks

Purified PCR amplicons were cloned into the pCR®4-TOPO vector for sequencing. The TOPO cloning reaction was carried out in a 200µl PCR tube containing 4 µl of PCR product, 1 µl salt solution and 1 µl of the TOPO vector. This was left for 30 minutes and stored on ice until transforming TOP10 cells with 2 µl of the reaction mixture. Cells were incubated for 10 min and heat shocked. Transformed cells were shaken at 37˚C for 1 hr before being plated onto LB agar plates containing Ampicillin (100 µg/ml) and allowed to grow overnight. Colonies were selected from two plates and grown overnight in LB broth containing Ampicillin (100 µg/ml). Plasmids were isolated from each culture

using a QIAprep Spin MiniPrep Kit (Qiagen) and a restriction digest was done using EcoRI overnight at 37˚C. The digested plasmids were run on a 1.5% agarose gel to identify successful cloning of the attb-TEV-Gene-attb PCR amplicons and confirmed by sequencing using the M13 forward and reverse primers that flank the PCR insert. The bacterial culture containing in frame attb-TEV-Gene-attb was stored in glycerol at -80˚C.

Gateway cloning and pilot expression

The entry vector was set up using the manufacturer's instructions. 5ul of the attb-TEV-GEne-attb PCR product was added to 1ul of pDONR and 2ul of TE pH 8.0. A positive control was done using 2ul of pEXP7-tet and 5ul of TE pH 8.0. Reactions were incubated for 1 hr at room temperature and then stopped using 1ul of proteinase K solution for 10 min at 37˚C. Transformation was done using 2ul of each reaction and 50ul of competent DH5α cells using identical procedure to the previously described TOPO cloning transformation. The cells were plated on LB agar plates containing 100ug/ml of kanamycin. Extracted plasmids were sequenced using the same M13 primers used previously.

Purified pDONR-MTase or pDONR-ParB (50-150ng) from the BP reaction was mixed with 1ul of pDEST17 and TE pH 8.0 for a total volume of 8ul per reaction. The reaction procedure was identical to the BP reaction (Figure 4.2). Transformation was done using 2ul of each reaction and 50ul of competent DH5α cells. The cells were plated on LB agar plates containing 100ug/ml of ampicillin. Extracted plasmids were sequenced prior to expression. Purified pDEST-MTase

isolated from DH5α cultures was used to transform BL-21-AI strain of *E. coli*. Cells were grown in 5ml of LB medium containing ampicillin until mid-log phase (OD_{600nm}≈0.5). 0.2% arabinose was added to induce expression. After 2hr induction 1.5ul samples were centrifuged for 1min at 14,000xg. Soluble protein was extracted from bacterial pellets using Qproteome Bacterial Protein Prep Kit (Qiagen). After centrifugation the supernatant containing soluble protein was placed in a new 1.5ml eppendorf tube and 200ul of lysis buffer (8M urea, 100mm NaH_2PO_4, 10mM Tris-Cl, 10mM imidazole, pH 8.0) was added to the remaining pellet which was agitated overnight at 37˚C. The lysates were centrifuged at 14,000xg for 30min at 4˚C. The supernatant containing any solubilized protein was transfered and placed in a new 1.5ml eppendorf tube.

The remaining pellets and 40ul aliquots of the soluble and insoluble fractions were resuspended in 40µl of 1x Laemmli sample buffer and heated for 10 min at 95˚C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done in 12.5% acrylamide separation gels with a 4% acrylamide stacking gel. Gels were poured into 12+2 well comb Criterion™ empty cassettes (Bio-Rad) and run in a Criterion™ gel electrophoresis cell, after 20µl of each sample was loaded into each lane, for 50 min at 200V. Protein bands were visualized using Coomassie brilliant blue R 250 staining overnight. This was followed by multiple washes with destain solution (30% acetic acid, 15% methanol).

Growth curve

In order to assess the viability of *E. coli* cells expressing either recombinant ParB or recombinant MTase, growth curves were generated. Growth of transformed bacterial cultures was performed at 37˚C for cultures containing either pDEST-MTase or pDEST-ParB. Fresh cultures of *E. coli* containing either pDEST-MTase or pDEST-ParB were started by innoculating 10µl of overnight culture in 5ml of LB broth containing 100µg/ml of ampicillin. Cultures were either left uninduced or induced 0.2% arabinose when OD=~0.4-0.5 and were grown at 37˚C for 7 hrs. Bacterial density was determined at hourly intervals by measuring OD at A_{600nm} in replicates of 5 using a Genesys 20 spectrophotometer (Thermo Scientific).

Results

Phylogenetic analysis of Drosophila ParB-MTase

Phylogenetic analysis of ParB-MTases from *Wolbachia*-infected *Drosophila* was performed in order to evaluate the evolutionary relationship of the genes and associated phages with their respective *Wolbachia* strains. Naming of ParB-MTases from *Wolbachia* is based on sequence similarity to the methyltransferase gene found in WOMelA (*met1*) and WOMelB (*met2*) (Saridaki et al., 2011) (Figure 4.3). Phylogenetic analysis of *met1* and *met2* amino acid sequences using ML methods reveals the seperation of the met1 genes from met2 (Figure 4.4) corresponding to the division of supergroup A and B of *Wolbachia*. The clustering

of the met2 gene is congruent with the accepted wsp phylogeny of associated *Wolbachia* strains.

Phylogenetic analysis of prokaryotic ParB-MTase

Prokaryotic ParB-MTase sequence data was derived mainly from whole-genome sequencing projects. The main source of the ParB-MTase sequences were from Gram negative Proteobacteria (67.8%); 47.5 % from alphaproteobacteria, and the Gram positive Firmicutes (23.5%). Phylogenetic analysis of 64 ParB-MTase amino acid sequences (Table 4.2) was performed in order to identify the evolutionary relationship of this protein. The phylogeny shows the separation of the bacterial phyla Firmicutes from the Proteobacteria, however within each of these clades the grouping of bacterial species belonging to different bacterial classes suggest the horizontal transfer of this gene (Figure 4.5).

Evaluating Phylogenetic Congruence

Horizontal gene transfer was evaluated by comparing tree topologies of ParB-MTase with 16S organismal phylogeny. Analysis was done on sequences from 10 species spread over five orders of alphaproteobacteria in order to statistically test for incongruence between these tree topologies. Horizontal gene transfer between these bacteria would be reflected by incongruency between the ParB-MTase and 16S topologies. Analysis of 16S ML phylogenetic tree resulted in little support for many of the nodes. There is agreement between the tree topology and published species trees for alphaproteobacteria (Williams et al., 2007). In the

published tree, Rhizobiales and Rhodobacteracea were sister groups, which is reflected in the 16S phylogeny with moderate bootstrap support (74%) (Figure 4.6). The lack of resolution to separate Rhodospirillales and Sphingomonadales may be due to the fact that there is only a single taxon from each order. In addition, Figure 4.6 is also in concordance with published phylogenies of alphaproteobacteria where the Rickettsiales order is basal to every other order in alphaproteobacteria (Williams et al., 2007).

Constraints were made on each tree topology corresponding to the highly supported clades found on the other tree. An optimal ML tree, without constraints was constructed. Additional separate ML trees for each constraint were generated. A score file containing the site-wise log-likelihoods was produced in PAUP* excluding gaps, and used for AU and SH in CONSEL. Comparing gene and organismal topologies, these constraints resulted in significant differences between the trees for all tests ($P<0.01$) and as a result the rejection of the constrained trees. The results of the AU and SH tests resulted in the rejection of the null hypothesis (the likelihoods of the topologies of both trees are not significantly different) and the acceptance of the alternative hypothesis (the topologies of both trees are significantly different) where ParB-MTases do not share evolutionary histories with their associated genome. The data suggests that the ParB-MTase has undergone horizontal gene transfer between species of alphaproteobacteria with the exception of *Nitrobacter sp Nb-311A* and *Nitrobacter hamburgensis*. In this case the monophyletic constraints resulted in a phylogeny that was not different from the consensus tree. In the case of

Nitrobacter this result is not surprising since the Rhizobiales order is highly supported in both trees. However although this constraint suggests that I cannot reject the alternative topologies based on the SH test, AU test ($P<0.05$). The AU test is similar to the SH test, such that it looks at a collection of trees and uses bootstrap resampling to create a distribution to correct for selection bias, however, it is less conservative than the SH test (Shiimodaira, 2002).

Comparative genome regions

For each ParB-MTase found in prokaryotic genomes, the gene neighborhood was analyzed. Examination of the gene location reveals that the ParB-MTase is usually associated with a prophage region in each of the taxa, and occurs upstream of terminase-portal protein systems (Figures 4.7, 4.8 and 4.9) with no observable shared gene organization of the regions upstream of the ParB-MTase between the bacteriophages (data not shown). The exceptions are *Clostridium tetani*, *Dahalococcoides ethenogenes* and *Streptococcus agalactiae* serotype III within the Firmicute clade and *Congregibacter litoralis, Hypomonas neptunium, Methylobacterium extorquens* and *Nitrobacter hambergenesis*. The ParB-MTase in each one of these genomes is within a region flanked by transposases or resolvases. This suggests that the location of these genes is the result of recombination or translocation.

In all the genomes examined ParB-MTase gene is generally associated with an annotated terminase gene (74.6%) and an associated annotated portal protein gene (60.3%). Based on this association the ParB-MTase is most likely involved in

translocation and head packaging of phage DNA. There are examples of shared regions between prophages with genetically similar ParB-MTase in different bacterial genera, as well as collinear prophage regions between genetically divergent ParB-MTase within the same genera. However there is no general consensus of gene synteny for prophages containing ParB-MTase. The *Wolbachia* ParB-MTases forms a single monophyletic clade. This clustering is similar to the *Wolbachia* phylogeny (Figure 4.5), suggesting a long association between the ParB-MTase, the associated WO phage, and respective *Wolbachia* strains.

Interestingly, the presence of two side by side ParB-MTase genes from two distinct clades is shared by six different genera of bacteria (Figures 4.8 and 4.9). One gene is a ParB-MTase with a DPPY catalytic site in motif IV of the MTase domain (Figure 4.9), whereas the other ParB-MTase has a SPPY catalytic site (Figure 4.8). These phages, which have collinear genomes probably resulted from horizontal transfer between their host bacteria, which now contain both a DPPY N6mA MTase and a SPPY N4mC MTase.

An examination of evolutionary relationship between the ParB-MTases and the terminase and portal proteins of prophages from 18 species of bacteria belonging to Firmicutes and Proteobacteria showed similar clustering of the portal proteins and terminase but not ParB-MTases (Figure 4.10).

Expression of recombinant ParB-MTase

The *Wolbachia* ParB and MTase subunits were cloned seperately into the pDEST17 vector in which ParB and MTase was fused to an N-terminal 6xHis-tag.

The expression of each recombinant protein was assayed (Figure 4.2). Both recombinant proteins were highly expressed in *E.coli* using the T7 promoter. Unfortunatly both recombinant proteins were insoluble, and were found only in the pellet after lysozyme treatment and centrifugation. Attempts to solubilize the MTase recombinant protein using 8M Urea were only partially successful, and downstream purification methods were unsuccessful in isolating this protein (data not shown).

Cultures expressing either ParB or MTase induced with 0.2% arabinose showed no significant change in growth rate (Figure 4.11) compared to uninduced cultures, indicating that *E. coli* cells expressing either one of these recombinant proteins were fully viable.

Discussion

In order to determine the relationship of the ParB-MTases in *Wolbachia*, a phylogenetic analysis was conducted. ParB-MTases from 11 different *Drosophila* species were labeled met1 or met2 corresponding to the naming of the MTase genes found in WOMelA or WOMelB respectively, and compared phylogenetically. Unlike other phage genes in *Wolbachia*, WO which show no concordance with *Wolbachia* strain (Biliske et al., 2011), the phylogenies of ParB-MTases, specifically the met2 gene shows congruence to the accepted wsp clustering of *Drosophila Wolbachia* strains. This suggests that the ParB-MTase gene and the prophage have a long and stable association with *Wolbachia*.

By examining *Wolbachia* strains that can rescue cytoplasmic incompatibility in *Drosophila*, Sardaki et al. (2011) demonstrated that a correlation exists between the expression of met2 and the rescue phenotype. Each CI inducing strain of *Wolbachia* has a functional and transcriptionally active met2 gene. There is however a difference in the rescue ability between A- and B-supergoup *Wolbachia* met2 genes. In A-group *Wolbachia*, the CI rescue phenotype occurs in strains that contain met2, and is absent in strains lacking a functional met2. This is not the case in B-group *Wolbachia* in which the rescue phenotype is associated with strains that have no functional met2 gene, suggesting that this supergoup may rely on a different mechanism for regulating CI (Sardaki et al., 2011). When screening multiple candidate genes for the regulation of CI, Yamada et al. (2011) expressed met2 in transgenic *D. melanogaster*; however this expression was unable to rescue the CI phenotype. These results suggest that the ParB-MTase is probably not the single gene responsible for this reproductive phenotype.

Ninety percent of all the sequenced ParB-MTases are found within Firmicutes (24%) or Proteobacteria (67%) genomes. Phylogenetic analysis of the ParB-MTase was done in order to determine the relationship of ParB-MTases. The ParB-MTase gene phylogeny (Figure 4.5) shows separate clustering of Gram-positive and Gram-negative bacteria. However, the phylogeny does not agree with published taxonomies of the Firmicute or Proteobacteria phyla which may indicate HT, or possibly recombination. The one exception to this is the *Wolbachia* ParB-MTases which represents a single monophyletic clade, suggesting that the gene was present in the common ancestor of *Wolbachia* A and

B supergroups, thus the long association of ParB-MTase with *Wolbachia* genomes. Extensive genome reduction has occurred in *Wolbachia* genomes suggests an essential role as all non-essential genes have been either lost or pseudogenized as a result of *Wolbachia's* intracellular lifestyle. Because of the genetic isolation of the *Wolbachia* clade, *Wolbachia* ParB-MTases are an exception to the horizontal transfer normally seen between distantly related Proteobacteria (Figure 4.5), and provide evidence of a long association between this gene and respective *Wolbachia* genomes.

The phylogeny suggests limited horizontal gene transfer of ParB-MTase across bacterial phyla but extensive transfer within bacterial phyla. The limited horizontal transfer between phyla is due to the nature of HGT which occurs more commonly between closely related organisms with restrictive host ranges. To examine the extent of horizontal transfer of the ParB-MTase alternative topologies between the 16S phylogeny of alphaproteobacteria and the ParB-MTase phylogeny were analyzed. Comparisons between optimal trees and alternative topologies where clades were constrained to be monophyletic revealed that the trees do not share an evolutionary history (Figure 4.6) indicating HGT of the ParB-MTase within alphaproteobacteria.

The genomic neighborhood of ParB-MTases in numerous prokaryote and phage genomes was examined in order to predict a possible functional role based on gene association. The position of the ParB-MTase gene in close association with the terminase and portal proteins suggests a role in bacteriophage DNA packaging. The terminase and portal proteins often occur as neighboring genes in

phage genomes (Burroughs et al., 2007). Together they are responsible for the DNA translocation involved in packaging phage DNA into procapsids during viral replication. This mechanism of DNA packaging is highly conserved among bacteriophages. DNA packaging is carried out by a terminase complex made up of subunits, the small and large terminase proteins. The small terminase subunit has a DNA-binding domain and is involved in binding specifically to phage DNA whereas the large subunit contains two domains, an endonuclease domain and an ATPase-containing translocase. Oligomers of the small terminase will initiate DNA packaging by binding to concatemeric phage DNA. This will form a complex with the large terminase subunit which cuts the phage DNA into genome-sized fragments prior to binding to the portal protein. During viral assembly, a dodecamer ring of portal protein forms on the procapsid. The large terminase packages the phage genome though the central channel of this ring into the procapsid (Sun et al., 2011).

It is unknown whether ParB-MTases have any role in this conserved bacteriophage DNA packaging system. Although phage associated MTases are part of R-M systems, as discussed below, solitary MTases involved in DNA packaging are uncommon. The only identified MTase involved in phage packaging is described for the bacteriophage P1 (Sternberg and Coulby, 1990). Unlike *Wolbachia* phages, the P1 bacteriophage exists as a plasmid and does not integrate into the genome. The P1 bacteriophage undergoes rolling-circle replication which generates a concatemer of the P1 genome. During DNA packaging the role of the P1 MTase is to methylate sites located on this

concatemer, known as *pac* sites (reviewed in Lobocka et al., 2004). These *pac* sites are cut prior to DNA packaging into the prohead and must be fully methylated for cleavage by the P1 packaging enzyme (pacase) to occur (Skorupski et al., 1994). Given that the bacteriophage P1 MTase is not homologous to the ParB-MTases examined in this study it is unlikely that the ParB-MTases have a related function despite their close association with the terminase and portal protein. However, their potential role in bacteriophage replication within *Wolbachia* should not be ruled out.

Given the mosaic nature of bacteriophage genomes resulting from widespread horizontal transfer (Hendrix et al., 1999) a phylogenetic analysis was performed in order to evaluate whether the ParB-MTases share an evolutionary history with the portal protein and terminases. Although the phylogenies of terminase and portal proteins share a similar evolutionary history, the incongruency between these genes and the ParB-MTase suggests that HGT of ParB-MTase occurs between bacteria within a single phylum and also among phages (Figure 4.10).

HGT is associated with MTases found in R-M systems of prokaryotes (Kobayashi, 2001). The mobility of these R-M systems results from their location near other mobile genetic elements such as bacteriophages or transposases. In prokaryotes, R-M systems exist as a defense against invading foreign DNA, such as bacteriophages. These systems are generally categorized into three different gene complexes (Roberts et al., 2003). The best characterized system is the Type II R-M, composed of a restriction (R) enzyme (REases), an endonuclease that will cleave specific sequences of DNA, and a separate modification (M) enzyme, an

MTase, that will methylated this same sequence. Methylation prevents REase activity thereby protecting the bacterial chromosomal DNA but cleaving invading unmethylated bacteriophage DNA. Within Type II R-M systems are subgroups of restriction enzymes composed of fused MTase and endonuclease domains within a single polypeptide (Roberts et al., 2003). Although the ParB-MTases examined in this study do not share any homology to these hybrid restriction enzymes their location within bacteriophages or in the case of *Clostridium tetani* (Figure 4.7) or *Xanthomonas albineans* (Figure 4.9) with transposases is a characterisitc also shared with Type II R-M systems.

Changes in DNA adenine methylation during the establishment of symbiosis occurs in the bacterial symbionts *Bradyrhizobium japonicum* and *Mesorhizobium loti* which are important for nitrogen fixation in host plant roots (Ichida et al., 2007). The methylation patterns on the bacterial genome changed during the bacterial-plant symbiotic interaction from the pattern seen in the non-symbiotic, free-living state, suggesting a change in the regulation of gene expression. The methylation status of *Wolbachia* DNA has not been determined. However, changes in host methylation as a result of a *Wolbachia* infection have been observed in *Aedes aegypti* where the presence of *Wolbachia* alters cytosine methylation in genes associated with membrane transport and communication (Ye et al., 2013). The feminizing *Wolbachia* strain found in the leafhopper *Zyginidia pullula* also alters the cytosine methylation patterns in males to resemble the pattern found in females which in turn alters gene expression required for sex differentiation (Negri et al., 2009). It is currently unknown how *Wolbachia*

mediates epigenetic cytosine modifications in the host genome. The role of adenine methylation in general and the ParB-MTase in the regulation of gene expression of essential endosymbiotic factors is under investigation. The presence of the *Wolbachia* ParB-MTase in egg cytoplasm of infected *Drosophila* embryos 15 minutes post fertilization (H. Braig and H. Harris, *personal communication*) provides a potential mechanism for the rescue of CI by adenine methylation of host DNA during first mitotic division.

The activity of the MTase domain of *Wolbachia* ParB-MTase from *D. simulans* could not be confirmed because the methylation site is currently not known. The dam-like methylation of GATC sites has been excluded for ParB-MTase with the use of methylation sensitive restriction enzymes (J. Haukedal, *personal communication*). Expression of separate ParB and MTase domains in *E.coli* was unsuccessful in determining nuclease or methyltransferase activity because expression of recombinant protein led to the formation of insoluble protein aggregates. Insoluble aggregates frequently occur when expressing recombinant proteins in a bacterial expression system (Frankel et al., 1991). This may not be a result of the properties the expressed bacteriophage protein. Overexpression of *E.coli* proteins for example the *E.coli* sigma subunit also results in the formation of insoluble aggregates (Gribskov and Burgess, 1983). In the case of ParB and MTase expressions, the use of chaotropic agents, e.g. urea, did not solubilize a sufficient amount of protein for downstream purification. Overexpression of either domain in *E. coli* resulted in no inhibition of cell growth,

indicating that these proteins are compatible with viability of *E. coli* cells. Alternatively, the insoubility of the recombinant proteins resulted in inactivation.

This study provides the groundwork for understanding the previously uncharacterized gene, ParB-MTase. The results demonstrate that the *Wolbachia* ParB-MTase is found primarily within prophages in a broad range of bacterial species. This gene has undergone HGT between bacteriophages and bacteria. This ParB-MTase may have a functional role in DNA packaging of capsid proteins based on the genomic neighborhood, but may have other roles in bacterial-insect symbiosis. Whether *Wolbachia* ParB-MTases act as solitary MTases or have a greater role in *Wolbachia*'s ability to establish and maintain endosymbiosis with a new host, warrants future investigations.

Tables

Table 4.1: BP Cloning Primers

Primer	Sequence	Tm (°C)
N1	5'-GAGAACCTGTACTTTCAGGGTATGAATTTAGCAATCCACTAC-3'	62.5
N2	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGGAGAACCTGTACTTTCAG -3'	67.6
C	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTATTATTGCTGTTTTTCTTCTTGAAT- 3'	66.3
N3	5'-GAGAACCTGTACTTTCAGGGTATGGCAGATATTACTGTTTGTGAT- 3'	63.9
C2	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTATTATTGATTTGCCAGTAACCGAAA- 3'	67.5

Table 4.2: ParB-MTase containing genomes used in this study

Organism/Species	Phylum;Class	Gene Name	UniProtKB Acession	Gene Ref Seq	Genome Ref Seq	Reference	Name
Natrialba phage PhiCh1	Myoviridae	PhiCh1p95	Q9T1P2	NP_666012	NC_004084	Klein et al. 2002	adenine methyltransferase
Elusimicrobium minutum (strain Pei191)	Elusimicrobia; Elusimicrobia	Emin_0959	B2KDB6	YP_001875849	NC_010644	Herlemann et al. 2009	DNA modification methylase
Hyphomonas neptunium (strain ATCC 15444)	Proteobacteria; Alphaproteobacteria	HNE_2545	Q0BZ55	YP_761238	NC_008358	Badger et al. 2006	DNA methylase
Congregibacter litoralis KT71	Proteobacteria; Gammaproteobacteria	KT71_18786	A4ACV0	ZP_01104405	NZ_CH672402	Fuchs et al. 2007	DNA methylase N-4/N-6
Nitrobacter hamburgensis (strain X14 / DSM 10229)	Proteobacteria; Alphaproteobacteria	Nham_0803	Q1QQ16	YP_576141	NC_007964	Starkenburg et al. 2008	DNA methylase N-4/N-6
Methylobacterium extorquens (strain DSM 5838 / DM4) (Methylobacterium dichloromethanicum (strain DM4)	Proteobacteria; Alphaproteobacteria	METDI0935	C7CCL6	YP_003066581	NC_012988	Vuilleumier et al. 2009	phage methyltransferase
Rhodobacter sphaeroides (strain KD131 / KCTC 12085)	Proteobacteria; Alphaproteobacteria	RSKD131_4069	B9KV51	YP_002521002	NC_011958	Lim et al. 2009	DNA modification methylase
Janthinobacterium sp. (strain Marseille) (Minibacterium massiliensis)	Proteobacteria; Betaproteobacteria;	mma_2700	A6T1J3	YP_001354390	NC_009659	Audic et al. 2007	DNA modification methylase
Ralstonia solanacearum (strain GMI1000) (Pseudomonas solanacearum)	Proteobacteria; Betaproteobacteria;	RSc0844	Q8Y149	NP_518965	NC_003295	Salanoubat et al. 2002	hypothetical protein RSc0844
Methylococcus capsulatus (strain ATCC 33009 / NCIMB 11132 / Bath)		MCA2654	Q603Z2	YP_115058	NC_002977	Ward et al. 2004	prophage LambdaMc01, DNA methyltransferase
Stenotrophomonas maltophilia (strain K279a)	Proteobacteria; Gammaproteobacteria	Smlt1882	B2FM38	YP_001971709	NC_010943	Crossman et al. 2008	DNA methylase
Acidovorax ebreus (strain TPSY) (Diaphorobacter sp. (strain TPSY))	Proteobacteria; Betaproteobacteria;	Dtpsy_3217	B9MH18	YP_002554646	NC_011992	Byrne-Bailey et al. 2010	DNA methylase n-4/n-6 domain-containing protein
Janthinobacterium sp. (strain Marseille) (Minibacterium massiliensis)	Proteobacteria; Betaproteobacteria	mma_2756	A6T1P9	YP_001354446	NC_009659	Audic et al. 2007	phage related DNA methyltransferase
Oligotropha carboxidovorans (strain ATCC 49405 / DSM 1227 / OM5)	Proteobacteria; Alphaproteobacteria	OCA5_c31320	B6JDM6	YP_004634057	NC_015684	Volland et al. 2011	DNA methylase
Magnetococcus sp. (strain MC-1)	Proteobacteria; Alphaproteobacteria	Mmc1_2753	A0LBA3	YP_866652	NC_008576	Schübbe et al. 2009	nuclease
Magnetococcus sp. (strain MC-1)	Proteobacteria; Alphaproteobacteria	Mmc1_2583	A0L786	YP_865235	NC_008576	Schübbe et al. 2009	nuclease
Magnetococcus sp. (strain MC-1)	Proteobacteria; Alphaproteobacteria	Mmc1_2825	A0LBH5	YP_866724	NC_008576	Schübbe et al. 2009	nuclease

Organism/Species	Phylum;Class	Gene Name	UniProtKB Acession	Gene Ref Seq	Genome Ref Seq	Reference	Name
Magnetococcus sp. (strain MC-1)	Proteobacteria; Alphaproteobacteria	Mmc1_2055	A0L9B3	YP_865962	NC_008576	Schübbe et al. 2009	nuclease
Magnetococcus sp. (strain MC-1)	Proteobacteria; Alphaproteobacteria	Mmc1_1249	A0L717	YP_865166	NC_008576	Schübbe et al. 2009	nuclease
Magnetococcus sp. (strain MC-1)	Proteobacteria; Alphaproteobacteria	Mmc1_2905	A0LBQ3	YP_866802	NC_008576	Schübbe et al. 2009	nuclease
Nitrobacter winogradskyi (strain Nb-255 / ATCC 25391)	Proteobacteria; Alphaproteobacteria	Nwi_1546	Q3SSD4	YP_318159	NC_007406	Starkenburg et al. 2006	DNA methylase N-4/N-6
Magnetospirillum magneticum (strain AMB-1 / ATCC 700264)	Proteobacteria; Alphaproteobacteria	amb0363	Q2WAF8	YP_419726	NC_007626	Matsunaga et al. 2005	modification methylase DpnIIB
Magnetospirillum magneticum (strain AMB-1 / ATCC 700264)	Proteobacteria; Alphaproteobacteria	amb1160	Q2W861	YP_420523	NC_007626	Matsunaga et al. 2005	modification methylase DpnIIB
Propionibacterium acnes (strain KPA171202 / DSM 16379)	Actinobacteridae	PPA1586	Q6A7D9	YP_056284	NC_006085	Brüggemann et al. 2004	ParB family DNA methylase
Lactobacillus gasseri (strain ATCC 33323 / DSM 20243)	Firmicutes; Bacilli	LGAS_1480	Q041N5	YP_815275	NC_008530	Makarova et al. 2006	ParB-like nuclease domain-containing protein
Lactobacillus reuteri	Firmicutes; Bacilli	lr0584	A5JJU0	ABQ44382	EF537905	Wall et al. 2007	ParB-like nuclease domain and DNA-modification methylase domain protein
Dehalococcoides ethenogenes (strain 195)	Chloroflexi; Dehalococcoidetes	DET0066	Q3ZAD1	YP_180818	NC_002936	Seshadri et al. 2005	DNA methylase
Bacillus cereus (strain ATCC 10987)	Firmicutes; Bacilli	BCE_0392	Q73EG6	NP_976720	NC_003909	Rasko et al. 2004	DNA methylase family protein
Clostridium kluyveri (strain ATCC 8527 / DSM 555 / NCIMB 10680)	Firmicutes; Clostridia	CKL_2649	A5N0L5	YP_001396032	NC_009706	Seedorf et al. unpublished	DNA methylase
Clostridium tetani (strain Massachusetts / E88)	Firmicutes; Clostridia	CTC01930	Q893A1	NP_782504	NC_004557	Brüggemann et al. 2003	chromosome partitioning parB family protein
Streptococcus pyogenes SpyM6 mefA phage-element	Firmicutes; Bacilli		Q6SZ19	AAR83216	AY445042	Banks et al. 2003	hypothetical protein
Streptococcus agalactiae serotype Ia (strain ATCC 27591 / A909 / CDC SS700)	Firmicutes; Bacilli;	SAK_0738	Q3K288	YP_329365	NC_007432	Tettelin et al. 2005	prophage LambdaSa04, DNA methylase
Streptococcus agalactiae CJB111	Firmicutes; Bacilli	SAM_2141	Q3DD78	ZP_00788405	NZ_AAJQ01000036	Tettelin et al. 2005	prophage LambdaW4, DNA methylase
Bacillus halodurans (strain ATCC BAA-125 / DSM 18197 / FERM 7344 / JCM 9153 / C-125)	Firmicutes; Bacilli;	BH3535	Q9K738	NP_244402	NC_002570	Takami et al. 2000	hypothetical protein BH3535
Listeria monocytogenes serotype 4b str. H7858	Firmicutes; Bacilli;	LMOh7858_pLM80_0040	Q4EJ05	ZP_00230385	NZ_AADR01000010	Nelson et al. 2004	adenine methyltransferase, putative

Organism/Species	Phylum;Class	Gene Name	UniProtKB Acession	Gene Ref Seq	Genome Ref Seq	Reference	Name
Enterococcus faecalis (strain ATCC 700802 / V583)	Firmicutes; Bacilli;	EF1452	Q835C5	NP_815173	NC_004668	Paulsen et al. 2003	adenine methyltransferase
Heliobacterium modesticaldum (strain ATCC 51547 / Ice1)	Firmicutes; Clostridia	HM1_2890	B0TCU8	YP_001681410	NC_010337	Sattley et al. 2008	DNA methylase family protein
Heliobacterium modesticaldum (strain ATCC 51547 / Ice1)	Firmicutes; Clostridia	HM1_2891	B0TCU9	YP_001681411	NC_010337	Sattley et al. 2008	DNA methylase
Heliobacterium modesticaldum (strain ATCC 51547 / Ice1)	Firmicutes; Clostridia	HM1_2946	B0TD04	YP_001681466	NC_010337	Sattley et al. 2008	DNA methylase
Dehalococcoides ethenogenes (strain 195)	Chloroflexi; Dehalococcoidetes	DET1092	Q3Z7J2	YP_181807	NC_002936	Seshadri et al. 2005	DNA methylase
Streptococcus phage EJ-1	Myoviridae	EJ-1p37	Q708N6	NP_945276	NC_005294	Romero et al. 2004	transferase
Streptococcus agalactiae serotype III (strain NEM316)	Firmicutes; Bacilli;	gbs1120	Q8E5B2	NP_735566	NC_004368	Glaser et al. 2002	hypothetical protein gbs1120
Streptococcus pyogenes serotype M1	Firmicutes; Bacilli;	SPy_0679	Q9A0P6	NP_268917	NC_002737	Ferretti et al. 2001	phage associated protein
Streptococcus equi subsp. equi (strain 4047)	Firmicutes; Bacilli	SEQ_0163	C0MBJ3	YP_002745581	NC_012471	Holden et al. 2009	phage DNA methylase
Enterococcus faecalis (strain ATCC 700802 / V583)	Firmicutes; Bacilli;	EF2114	Q832V4	NP_815777	NC_004668	Paulsen et al. 2003	adenine methyltransferase
Fusobacterium nucleatum subsp. vincentii ATCC 49256	Fusobacteria; Fusobacteriales	FNV2234	Q7P8F6	ZP_00143186	NZ_AABF02000002	Kapatral et al. 2003	Chromosome partitioning protein parB
Desulfovibrio vulgaris (strain Hildenborough / ATCC 29579 / NCIMB 8303)	Proteobacteria; Deltaproteobacteria	DVU2878	Q727H7	YP_012090	NC_002937	Heidelberg et al. 2004	adenine specific DNA methyltransferase
Desulfovibrio vulgaris (strain Hildenborough / ATCC 29579 / NCIMB 8303)	Proteobacteria; Deltaproteobacteria	DVU0192	Q72FM1	YP_009417	NC_002937	Heidelberg et al. 2004	adenine specific DNA methyltransferase
Janthinobacterium sp. (strain Marseille) (Minibacterium massiliensis)	Proteobacteria; Betaproteobacteria	mma_2757	A6T1Q0	YP_001354447	NC_009659	Audic et al. 2007	DNA modification methylase
Acidovorax ebreus (strain TPSY) (Diaphorobacter sp. (strain TPSY))	Proteobacteria; Betaproteobacteria	Dtpsy_3216	B9MH17	YP_002554645	NC_011992	Byrne-Bailey et al. 2010	DNA methylase n-4/n-6 domain-containing protein
Stenotrophomonas maltophilia (strain K279a)	Proteobacteria; Gammaproteobacteria	Smlt1881	B2FM37	YP_001971708	NC_010943	Crossman et al. 2008	DNA methyltransferase
Janthinobacterium sp. (strain Marseille) (Minibacterium massiliensis)	Proteobacteria; Betaproteobacteria	mma_2701	A6T1J4	YP_001354391	NC_009659	Audic et al. 2007	phage related DNA methyltransferase
Methylococcus capsulatus (strain ATCC 33009 / NCIMB 11132 / Bath)	Proteobacteria; Gammaproteobacteria	MCA2655	Q603Z1	YP_115059	NC_002977	Ward et al. 2004	prophage LambdaMc01, DNA methyltransferase
Ralstonia solanacearum (strain GMI1000) (Pseudomonas solanacearum)	Proteobacteria; Betaproteobacteria	RSc0845	Q8Y148	NP_518966	NC_003295	Salanoubat et al. 2002	hypothetical protein RSc0845

Organism/Species	Phylum;Class	Gene Name	UniProtKB Acession	Gene Ref Seq	Genome Ref Seq	Reference	Name
Acidithiobacillus caldus ATCC 51756	Proteobacteria; Gammaproteobacteria	ACA_2137	C6NTP6	ZP_05292387	NZ_ACVD01000058	Valdes et al. 2009	DNA methylase
Hahella chejuensis (strain KCTC 2396)	Proteobacteria; Gammaproteobacteria	HCH_05682	Q2SAI7	YP_436762	NC_007645	Jeong et al. 2005	DNA modification methylase
Xanthomonas albilineans (strain GPE PC73 / CFBP 7063)	Proteobacteria; Gammaproteobacteria	XALc_2626	D2UFE3	YP_003377098	NC_013722	Pieretti et al. 2009	DNA modification methylase
Xanthomonas albilineans (strain GPE PC73 / CFBP 7063)	Proteobacteria; Gammaproteobacteria	XALc_2394	D2U9D2	YP_003376866	NC_013722	Pieretti et al. 2009	adn methyltransferase
Magnetospirillum gryphiswaldense	Proteobacteria; Alphaproteobacteria	MGR_2343	A4TYQ9	CAM75766	CU459003	Richter et al. 2007	DNA methylase N-4/N-6:ParB-like nuclease
Wolbachia sp. subsp. Drosophila simulans (strain wRi)	Proteobacteria; Alphaproteobacteria	WRi_010300	C0R349		NC_012416	Klasson et al. 2009	DNA methylase
Wolbachia pipientis wMel	Proteobacteria; Alphaproteobacteria	WD_0594	Q05HL6	NP_966363	NC_002978	Wu et al. 2004	prophage LambdaW4, DNA methylase
Wolbachia endosymbiont of Culex quinquefasciatus JHB	Proteobacteria; Alphaproteobacteria	C1A_1204	B6Y9A3	ZP_03335516	NZ_DS996942	Puiu et al. unpublished	Phage related DNA methylase
Wolbachia pipientis subsp. Culex pipiens (strain wPip)	Proteobacteria; Alphaproteobacteria	WP0429	B3CPN4	YP_001975212	NC_010981	Klasson et al. 2008	Phage related DNA methylase
Wolbachia pipientis wMel	Proteobacteria; Alphaproteobacteria	WD_0263	Q05HL9	NP_966069	NC_002978	Wu et al. 2004	prophage LambdaW1, DNA methylase

Figures

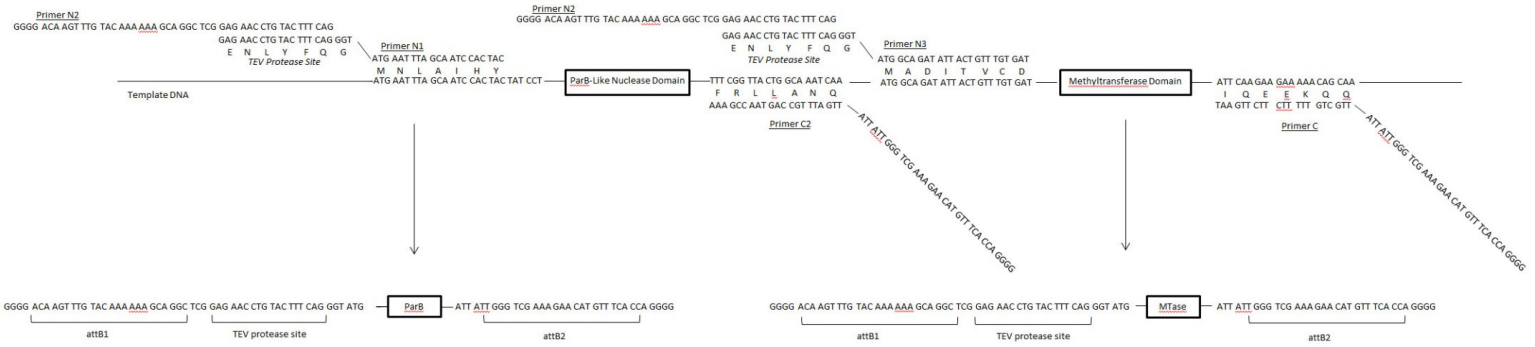


Figure 4.1. Schematic of primer design for the amplification of seperate ParB and MTase domains for Gateway cloning. Adapted from a Nallamsetty and Waugh (2007).

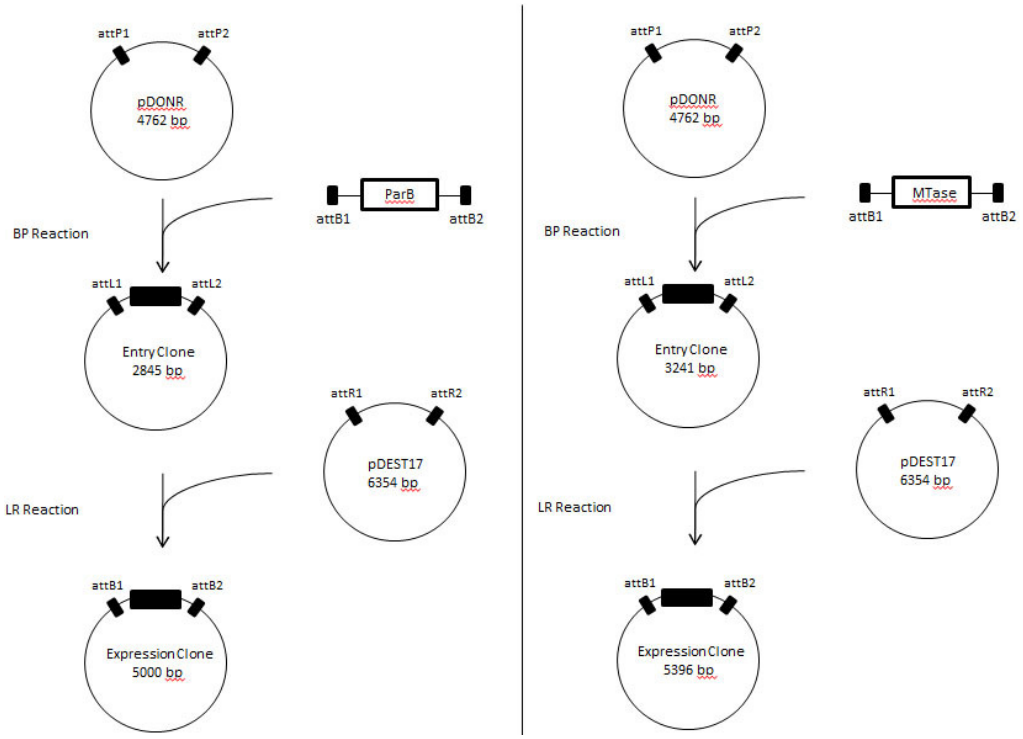


Figure 4.2. Schematic of gateway cloning of ParB and MTase domains. The PCR amplicon of each domain with attB sites is cloned into an entry vector through the BP reaction. The transfer of this insertion into an expression clone is done through the LR reaction with a destination vector.

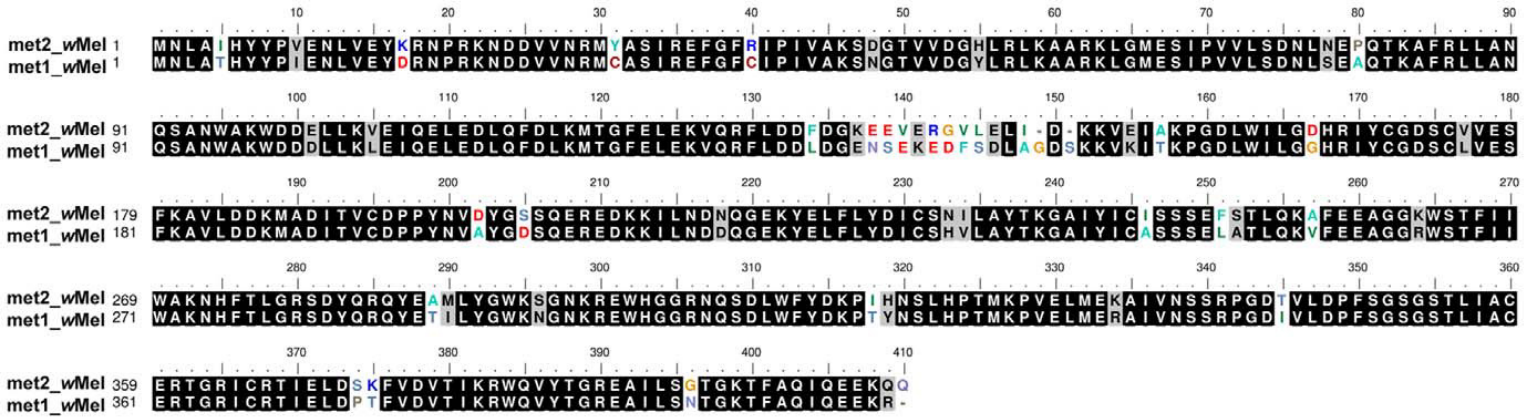


Figure 4.3. Amino acid alignment of Met1 and Met2 proteins of *Wolbachia* strain wMel. Black highlight indicates amino acid identity; greyhighlight indicates amino acid similarity.

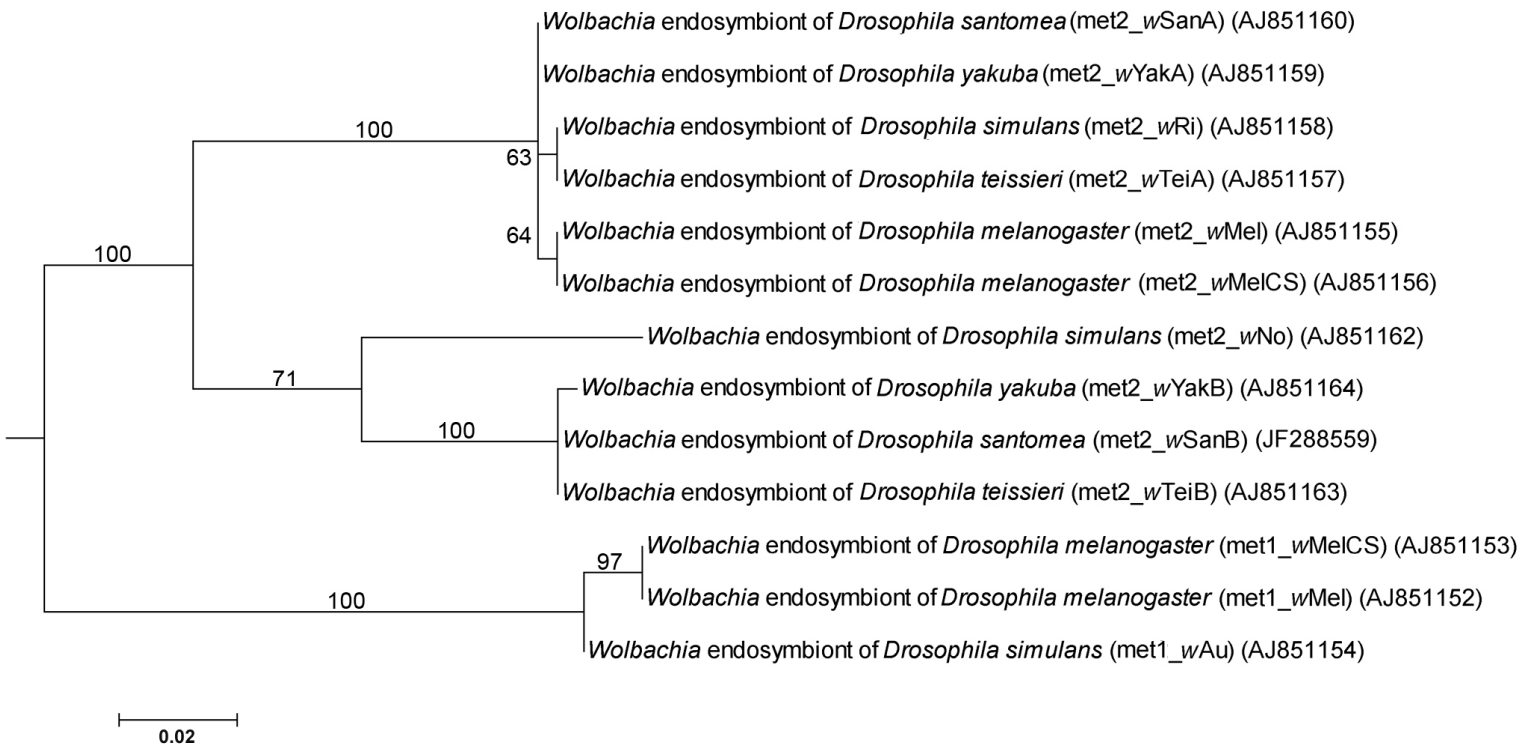


Figure 4.4. Phylogenetic tree of *Wolbachia* MTase (met1 and met2) gene sequences found in *Drosophila*. The tree was constructed by Maximum Likelihood analysis. Numbers on the nodes indicate bootstrap values.

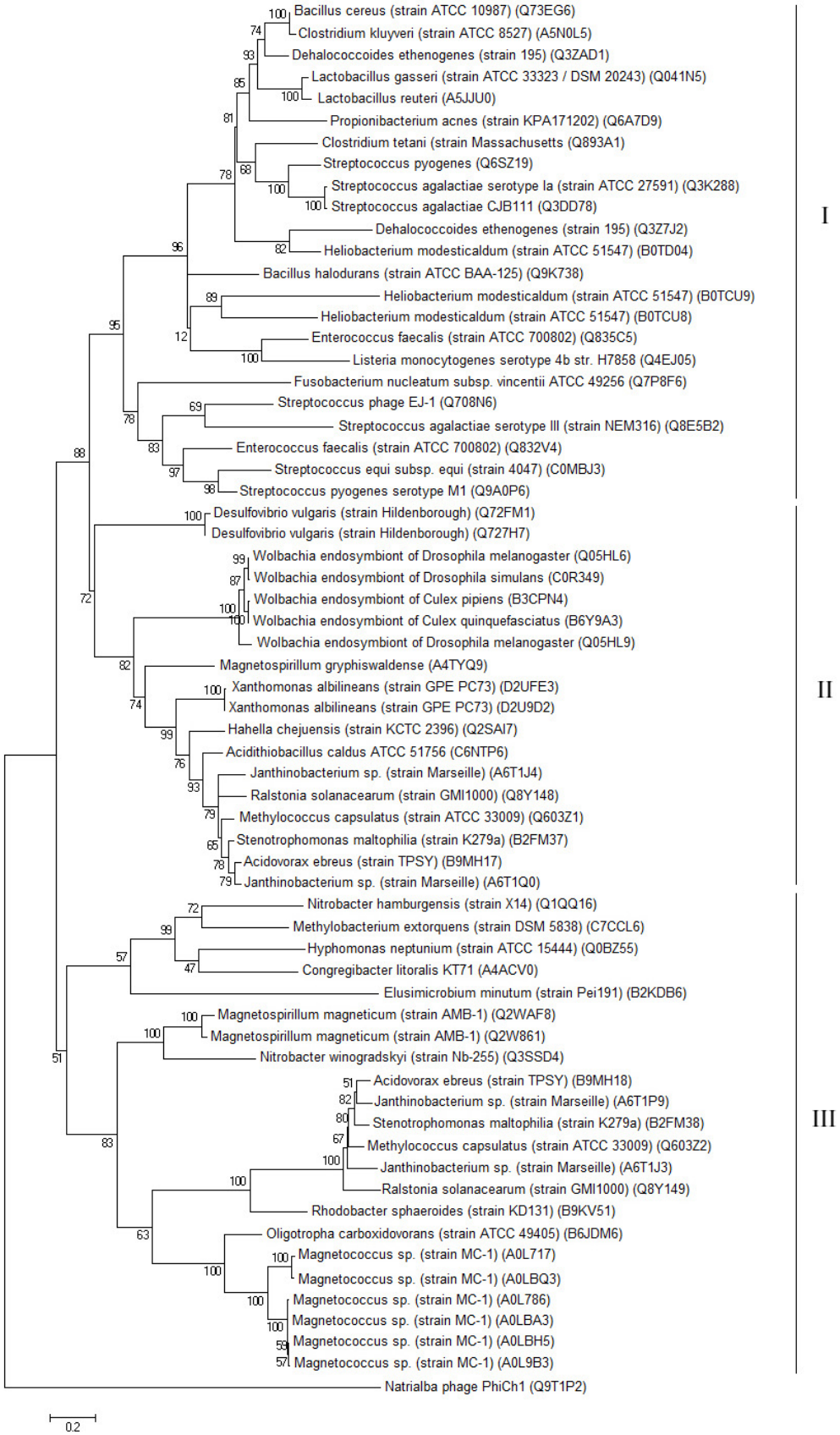

Bacillus cereus (strain ATCC 10987) (Q73EG6)
Clostridium kluyveri (strain ATCC 8527) (A5N0L5)
Dehalococcoides ethenogenes (strain 195) (Q3ZAD1)
Lactobacillus gasseri (strain ATCC 33323 / DSM 20243) (Q041N5)
Lactobacillus reuteri (A5JJU0)
Propionibacterium acnes (strain KPA171202) (Q6A7D9)
Clostridium tetani (strain Massachusetts (Q893A1)
Streptococcus pyogenes (Q6SZ19)
Streptococcus agalactiae serotype Ia (strain ATCC 27591) (Q3K288)
Streptococcus agalactiae CJB111 (Q3DD78)
Dehalococcoides ethenogenes (strain 195) (Q3Z7J2)
Heliobacterium modesticaldum (strain ATCC 51547) (B0TD04)
Bacillus halodurans (strain ATCC BAA-125) (Q9K738)
Heliobacterium modesticaldum (strain ATCC 51547) (B0TCU9)
Heliobacterium modesticaldum (strain ATCC 51547) (B0TCU8)
Enterococcus faecalis (strain ATCC 700802) (Q835C5)
Listeria monocytogenes serotype 4b str. H7858 (Q4EJ05)
Fusobacterium nucleatum subsp. vincentii ATCC 49256 (Q7P8F6)
Streptococcus phage EJ-1 (Q708N6)
Streptococcus agalactiae serotype III (strain NEM316) (Q8E5B2)
Enterococcus faecalis (strain ATCC 700802) (Q832V4)
Streptococcus equi subsp. equi (strain 4047) (C0MBJ3)
Streptococcus pyogenes serotype M1 (Q9A0P6)
Desulfovibrio vulgaris (strain Hildenborough) (Q72FM1)
Desulfovibrio vulgaris (strain Hildenborough) (Q727H7)
Wolbachia endosymbiont of Drosophila melanogaster (Q05HL6)
Wolbachia endosymbiont of Drosophila simulans (C0R349)
Wolbachia endosymbiont of Culex pipiens (B3CPN4)
Wolbachia endosymbiont of Culex quinquefasciatus (B6Y9A3)
Wolbachia endosymbiont of Drosophila melanogaster (Q05HL9)
Magnetospirillum gryphiswaldense (A4TYQ9)
Xanthomonas albilineans (strain GPE PC73) (D2UFE3)
Xanthomonas albilineans (strain GPE PC73) (D2U9D2)
Hahella chejuensis (strain KCTC 2396) (Q2SAI7)
Acidithiobacillus caldus ATCC 51756 (C6NTP6)
Janthinobacterium sp. (strain Marseille) (A6T1J4)
Ralstonia solanacearum (strain GMI1000) (Q8Y148)
Methylococcus capsulatus (strain ATCC 33009) (Q603Z1)
Stenotrophomonas maltophilia (strain K279a) (B2FM37)
Acidovorax ebreus (strain TPSY) (B9MH17)
Janthinobacterium sp. (strain Marseille) (A6T1Q0)
Nitrobacter hamburgensis (strain X14) (Q1QQ16)
Methylobacterium extorquens (strain DSM 5838) (C7CCL6)
Hyphomonas neptunium (strain ATCC 15444) (Q0BZ55)
Congregibacter litoralis KT71 (A4ACV0)
Elusimicrobium minutum (strain Pei191) (B2KDB6)
Magnetospirillum magneticum (strain AMB-1) (Q2WAF8)
Magnetospirillum magneticum (strain AMB-1) (Q2W861)
Nitrobacter winogradskyi (strain Nb-255) (Q3SSD4)
Acidovorax ebreus (strain TPSY) (B9MH18)
Janthinobacterium sp. (strain Marseille) (A6T1P9)
Stenotrophomonas maltophilia (strain K279a) (B2FM38)
Methylococcus capsulatus (strain ATCC 33009) (Q603Z2)
Janthinobacterium sp. (strain Marseille) (A6T1J3)
Ralstonia solanacearum (strain GMI1000) (Q8Y149)
Rhodobacter sphaeroides (strain KD131) (B9KV51)
Oligotropha carboxidovorans (strain ATCC 49405) (B6JDM6)
Magnetococcus sp. (strain MC-1) (A0L717)
Magnetococcus sp. (strain MC-1) (A0LBQ3)
Magnetococcus sp. (strain MC-1) (A0L786)
Magnetococcus sp. (strain MC-1) (A0LBA3)
Magnetococcus sp. (strain MC-1) (A0LBH5)
Magnetococcus sp. (strain MC-1) (A0L9B3)
Natrialba phage PhiCh1 (Q9T1P2)
0.2
I
II
III

Figure 4.5 Phylogenetic tree of bacterial ParB-MTases sequences retrieved from PIR. Tree of highest likelihood is shown. Clades representing Firmicutes (I) and Proteobacteria (II and III) are labeled. Tree is midpoint rooted with bootstrap values for nodes are listed for maximum likelihood analysis.

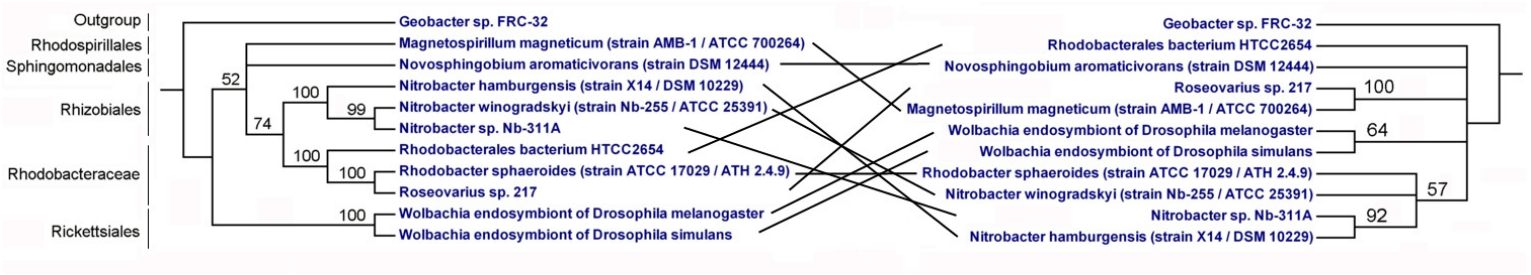


Figure 4.6: Maximul likelihood phylogenetic tree of organismal phylogeny (based on 16S sequence data) of alphaproteobacteria (left) and of associated ParB-MTase (right).

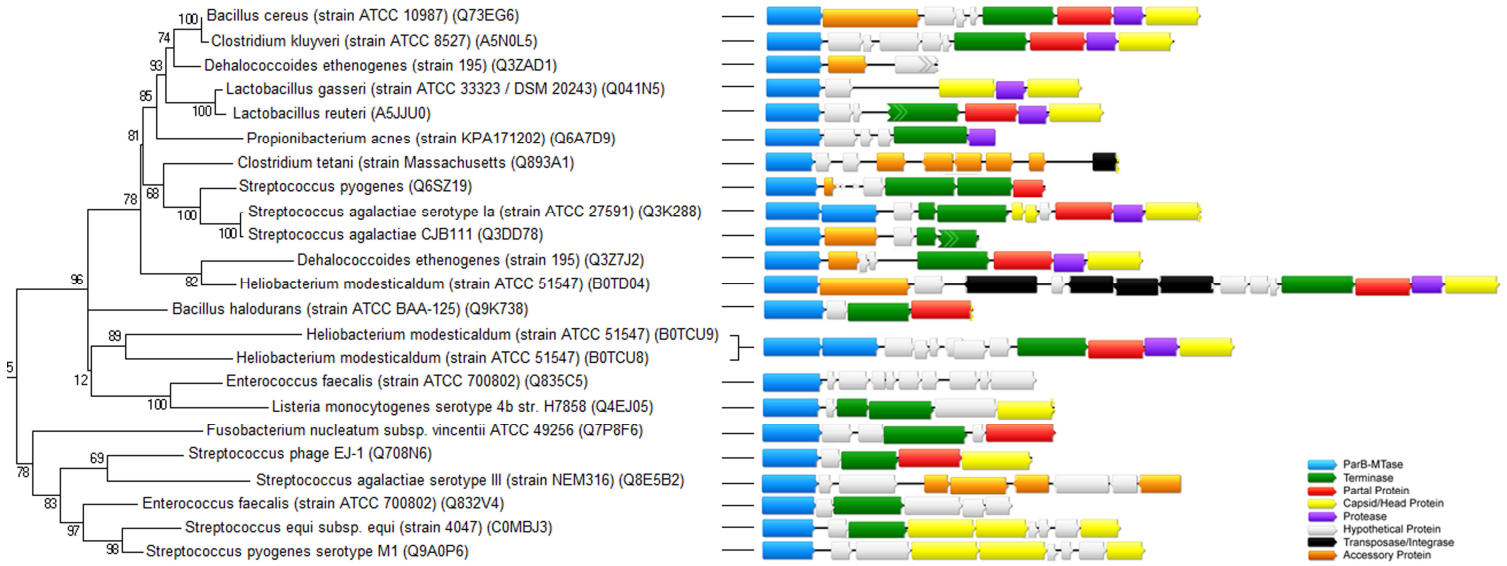


Figure 4.7: Gene neighborhood of ParB-MTase in Firmicutes (I). Neighborhoods are aligned at the ParB-MTase genes (blue) for each bacterial species. Size of each bar corresponds to gene size, arrowhead indicates orientation.

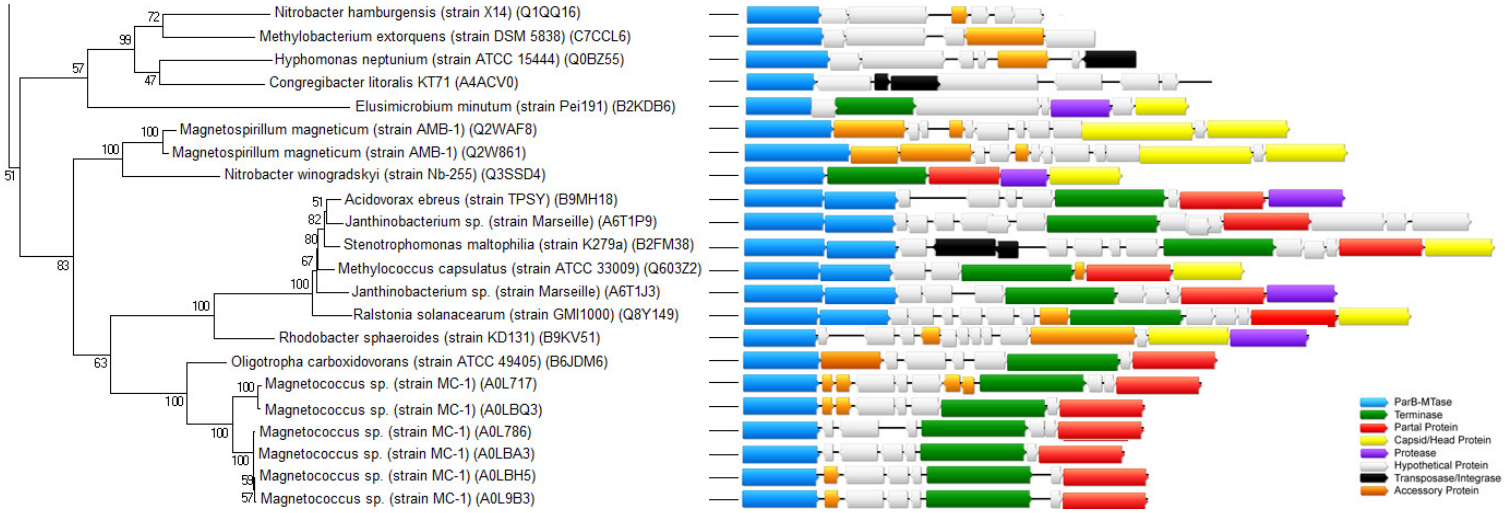


Figure 4.8: Gene neighborhood of ParB-MTase in Proteobacteria clade (II). Neighborhoods are aligned at the ParB-MTase genes (blue) for each bacterial species. Size of each bar corresponds to gene size, arrowhead indicates orientation.

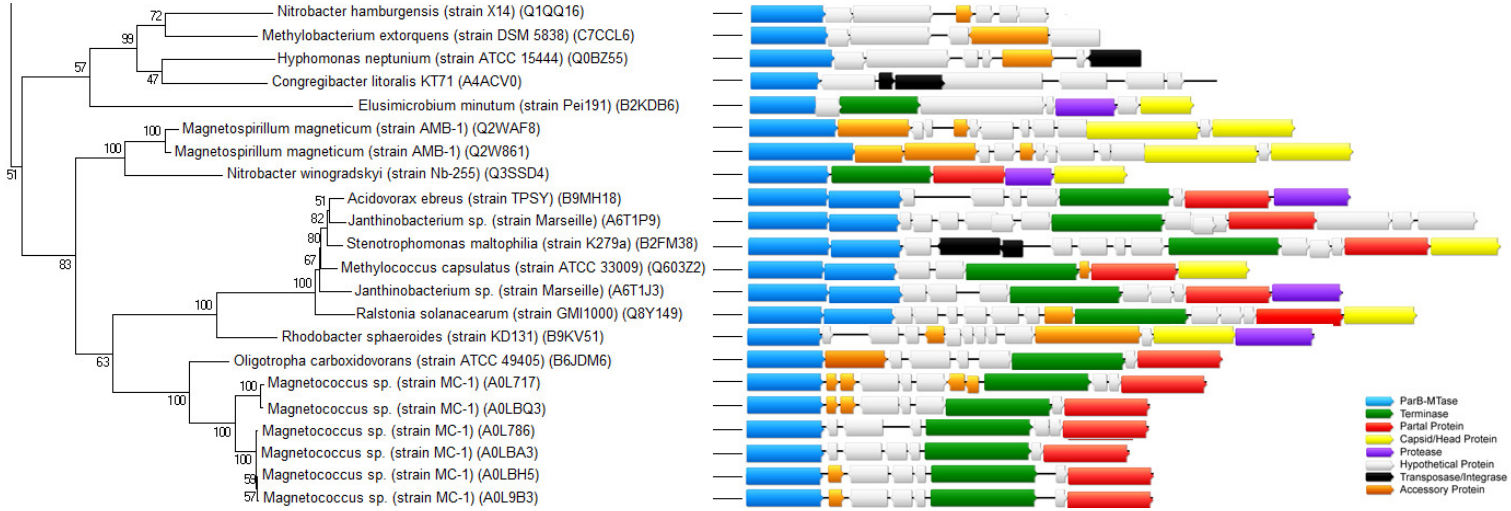
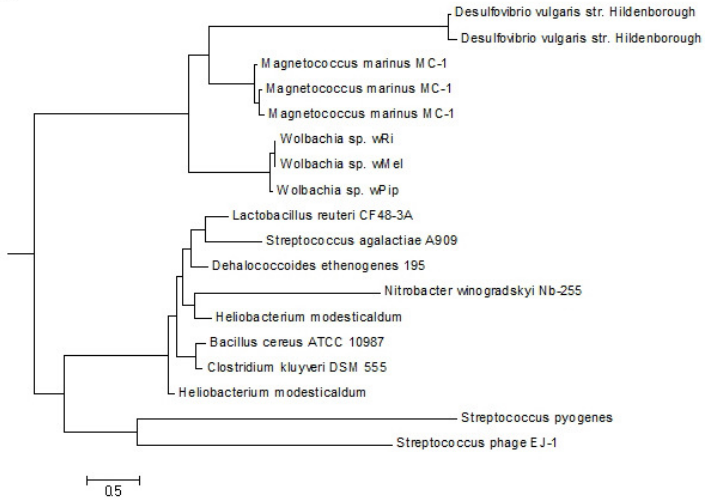
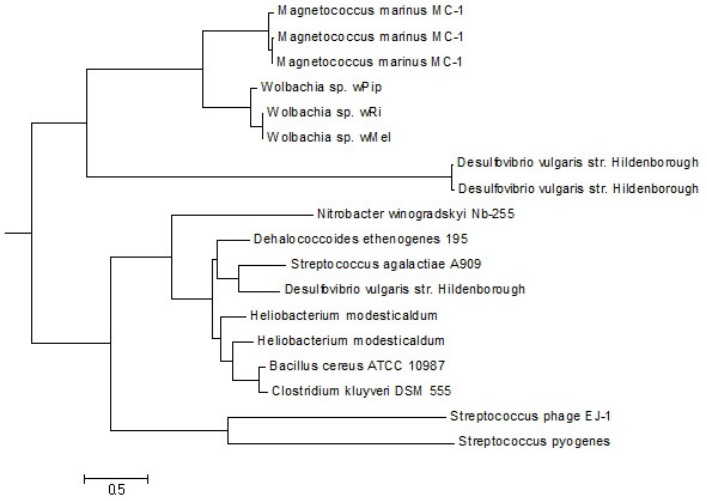


Figure 4.9: Gene neighborhood of ParB-MTase in Proteobacteria (III). Neighborhoods are aligned at the ParB-MTase genes (blue) for each bacterial species. Size of each bar corresponds to gene size, arrowhead indicates orientation.

A


Desulfovibrio vulgaris str. Hildenborough
Desulfovibrio vulgaris str. Hildenborough
Magnetococcus marinus MC-1
Magnetococcus marinus MC-1
Magnetococcus marinus MC-1
Wolbachia sp. wRi
Wolbachia sp. wMel
Wolbachia sp. wPip
Lactobacillus reuteri CF48-3A
Streptococcus agalactiae A909
Dehalococcoides ethenogenes 195
Nitrobacter winogradskyi Nb-255
Heliobacterium modesticaldum
Bacillus cereus ATCC 10987
Clostridium kluyveri DSM 555
Heliobacterium modesticaldum
Streptococcus pyogenes
Streptococcus phage EJ-1
0.5

B


Magnetococcus marinus MC-1
Magnetococcus marinus MC-1
Magnetococcus marinus MC-1
Wolbachia sp. wPip
Wolbachia sp. wRi
Wolbachia sp. wMel
Desulfovibrio vulgaris str. Hildenborough
Desulfovibrio vulgaris str. Hildenborough
Nitrobacter winogradskyi Nb-255
Dehalococcoides ethenogenes 195
Streptococcus agalactiae A909
Desulfovibrio vulgaris str. Hildenborough
Heliobacterium modesticaldum
Heliobacterium modesticaldum
Bacillus cereus ATCC 10987
Clostridium kluyveri DSM 555
Streptococcus phage EJ-1
Streptococcus pyogenes
0.5

C

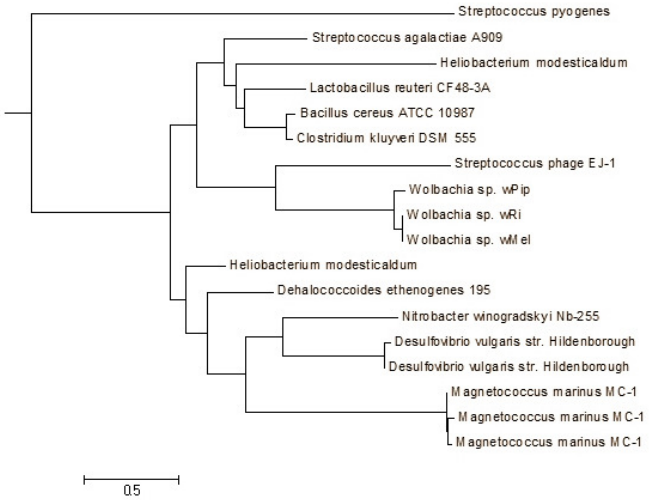

Streptococcus pyogenes
Streptococcus agalactiae A909
Heliobacterium modesticaldum
Lactobacillus reuteri CF48-3A
Bacillus cereus ATCC 10987
Clostridium kluyveri DSM 555
Streptococcus phage EJ-1
Wolbachia sp. wPip
Wolbachia sp. wRi
Wolbachia sp. wMel
Heliobacterium modesticaldum
Dehalococcoides ethenogenes 195
Nitrobacter winogradskyi Nb-255
Desulfovibrio vulgaris str. Hildenborough
Desulfovibrio vulgaris str. Hildenborough
Magnetococcus marinus MC-1
Magnetococcus marinus MC-1
Magnetococcus marinus MC-1
0.5

Figure 4.10: Evolutionary relationship of genomic neighborhoods of ParB-MTases in bacteria from Proteobacteria and Firmicutes. Maximum likelihood phylogenetic trees are shown for (A) Terminase (B) Portal protein and (C) ParB-MTase. Each tree is midpoint rooted.

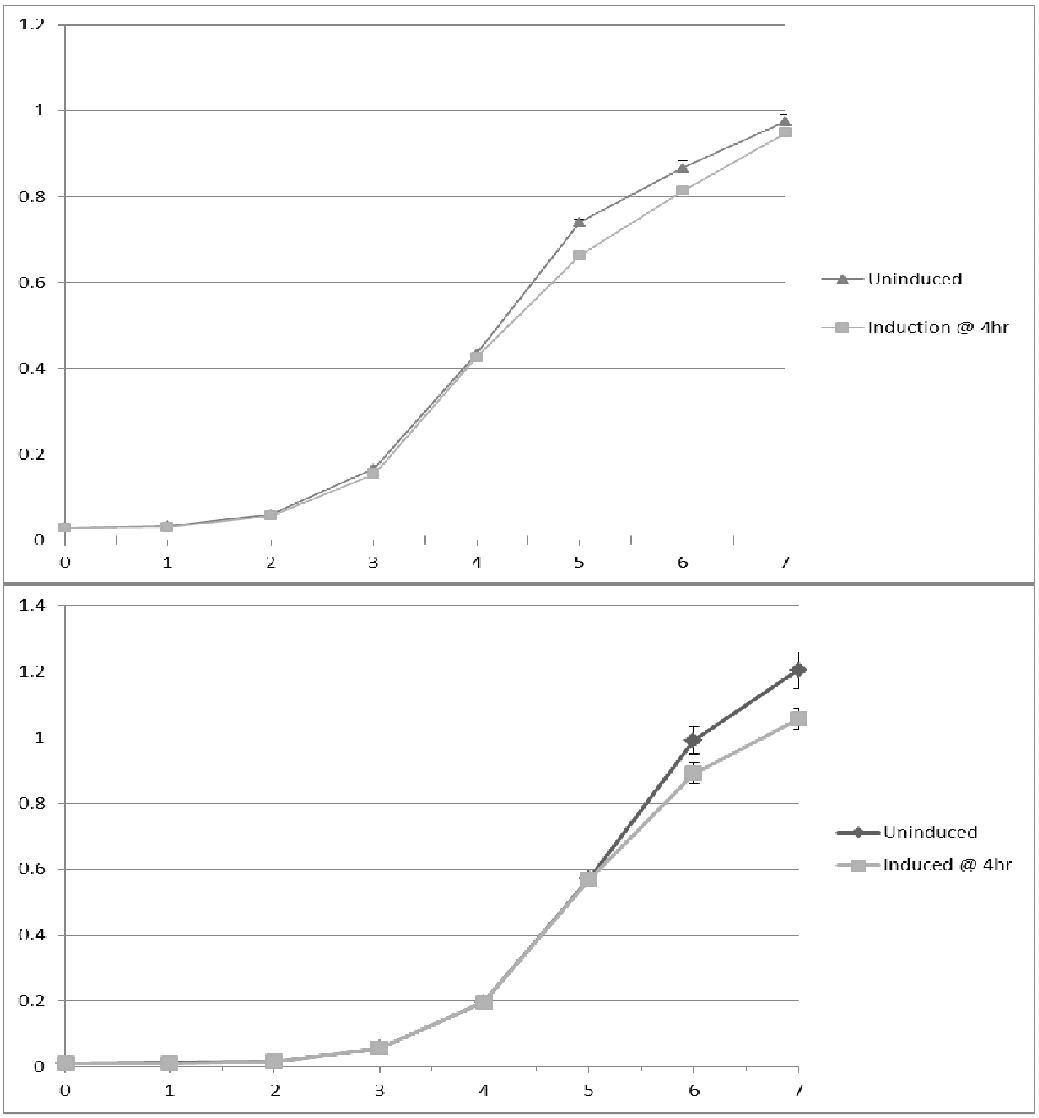


Figure 4.11: Growth of *E. coli* following expression of the (A) ParB domain and (B) MTase domain from *Wolbachia* wRi in *E.coli*. *E.coli* with plasmids pDEST-ParB or pDEST-MTase was grown in LB broth at 37˚C to an OD_{600} of ~0.4 before induction with 0.2% arabinose.

References:

Abascal F, Zardoya R, Posada, D. 2005. ProtTest: Selection of best-fit models of protein evolution. Bioinformatics, 21(9):2104-2105.

Audic S, Robert C, Campagna B, Parinello H, Claverie JM, Raoult D, Drancourt M. 2007. Genome analysis of *Minibacterium massiliensis* highlights the convergent evolution of water-living bacteria. PLoS Genetics, 3(8):e138.

Badger JH, Hoover TR, Brun YV, Weiner RM, Laub MT, Alexandre G, Mrázek J, Ren Q, Paulsen IT, Nelson KE, Khouri HM, Radune D, Sosa J, Dodson RJ, Sullivan SA, Rosovitz MJ, Madupu R, Brinkac LM, Durkin AS, Daugherty SC, Kothari SP, Giglio MG, Zhou L, Haft DH, Selengut JD, Davidsen TM, Yang Q, Zafar N, Ward NL. 2006. Comparative genomic evidence for a close relationship between the dimorphic prosthecate bacteria *Hyphomonas neptunium* and *Caulobacter crescentus*. Journal of Bacteriology, 188(19):6841-50.

Banks DJ, Porcella SF, Barbian KD, Martin JM, Musser JM. 2003. Structure and distribution of an unusual chimeric genetic element encoding macrolide resistance in phylogenetically diverse clones of group A *Streptococcus.* Journal of Infectious Diseases, 188(12):1898-908.

Biliske JA, Batista PD, Grant CL, Harris HL. 2011. The bacteriophage WORiC is the active phage element in wRi of *Drosophila simulans* and represents a conserved class of WO phages. BMC Microbiology, 11:251.

Brüggemann H, Bäumer S, Fricke WF, Wiezer A, Liesegang H, Decker I, Herzberg C, Martinez-Arias R, Merkl R, Henne A, Gottschalk G. 2003. The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. Proceedings of the National Academy of Sciences USA, 100(3):1316-21.

Brüggemann H, Henne A, Hoster F, Liesegang H, Wiezer A, Strittmatter A, Hujer S, Dürre P, Gottschalk G. 2004. The complete genome sequence of *Propionibacterium acnes*, a commensal of human skin. Science, 305(5684):671-3. -9.

Bujnicki JM, Radlinska M. 1999. Molecular evolution of DNA-(cytosine-N4) methyltransferases: evidence for their polyphyletic origin. Nucleic Acids Research, 27(22):4501-9.

Burroughs AM, Iyer LM, Aravind L. 2007. Comparative genomics and evolutionary trajectories of viral ATP dependent DNA-packaging systems. Genome Dynamics, 3:48-65.

Byrne-Bailey KG, Weber KA, Chair AH, Bose S, Knox T, Spanbauer TL, Chertkov O, Coates JD. 2010. Completed genome sequence of the anaerobic iron-oxidizing bacterium *Acidovorax ebreus* strain TPSY. Journal of Bacteriology, 192(5):1475-6.

Casadesús J, Low D. 2006. Epigenetic gene regulation in the bacterial world. Microbiology and Molecular Biology Reviews, 70(3):830-56.

Cheng X. 1995. Structure and function of DNA methyltransferases. Annual Review of Biophysics and Biomolecular Structure..;24:293-318.

Collier J, McAdams HH, Shapiro L. 2007. A DNA methylation ratchet governs progression through a bacterial cell cycle. Proceedings of the National Academy of Sciences USA, 104(43):17111-6.

Crossman LC, Gould VC, Dow JM, Vernikos GS, Okazaki A, Sebaihia M, Saunders D, Arrowsmith C, Carver T, Peters N, Adlem E, Kerhornou A, Lord A, Murphy L, Seeger K, Squares R, Rutter S, Quail MA, Rajandream MA, Harris D, Churcher C, Bentley SD, Parkhill J, Thomson NR, Avison MB. 2008. The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. Genome Biology, 9(4):R74.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods, 9(8):772.

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A. 2011. Geneious. Version 5.4. http://www.geneious.com.

Drummond AJ, Ashton B, Cheung M, Heled J, Kearse M, Moir R, Stones-Havas S, Thierer T, Wilson A. 2010. Geneious. Version 5.3. http://www.geneious.com

Ferretti JJ, McShan WM, Ajdic D, Savic DJ, Savic G, Lyon K, Primeaux C, Sezate S, Suvorov AN, Kenton S, Lai HS, Lin SP, Qian Y, Jia HG, Najar FZ, Ren Q, Zhu H, Song L, White J, Yuan X, Clifton SW, Roe BA, McLaughlin R. 2001. Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. Proceedings of the National Academy of Sciences USA, 98(8):4658-63.

Frankel S, Sohn R, Leinwand L. 1991. The use of sarkosyl in generating soluble protein after bacterial expression. Proceedings of the National Academy of Sciences USA, 88(4):1192-6.

Fuchs BM, Spring S, Teeling H, Quast C, Wulf J, Schattenhofer M, Yan S, Ferriera S, Johnson J, Glöckner FO, Amann R. 2007. Characterization of a marine gammaproteobacterium capable of aerobic anoxygenic photosynthesis. Proceedings of the National Academy of Sciences USA, 104(8):2891-6.

Glaser P, Rusniok C, Buchrieser C, Chevalier F, Frangeul L, Msadek T, Zouine M, Couvé E, Lalioui L, Poyart C, Trieu-Cuot P, Kunst F. 2002. Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. Molecular Microbiology, 45(6):1499-513.

Gribskov M, Burgess RR. 1983. Overexpression and purification of the sigma subunit of *Escherichia coli* RNA polymerase. Gene, 26(2-3):109-18.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology, 52: 696-704.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41:95-98.

Herlemann DP, Geissinger O, Ikeda-Ohtsubo W, Kunin V, Sun H, Lapidus A, Hugenholtz P, Brune A. 2009. Genomic analysis of "*Elusimicrobium minutum*," the first cultivated representative of the phylum "Elusimicrobia" (formerly termite group 1). Applied and Environmental Microbiology, 75(9):2841-9.

Hendrix RW, Smith MC, Burns RN, Ford ME, Hatfull GF. 1999. Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. Proceedings of the National Academy of Sciences USA, 96(5):2192-7.

Heusipp G, Fälker S, Schmidt MA. 2007. DNA adenine methylation and bacterial pathogenesis. International Journal of Medical Microbiology, 297(1):1-7.

Holden MT, Heather Z, Paillot R, Steward KF, Webb K, Ainslie F, Jourdan T, Bason NC, Holroyd NE, Mungall K, Quail MA, Sanders M, Simmonds M, Willey D, Brooks K, Aanensen DM, Spratt BG, Jolley KA, Maiden MC, Kehoe M, Chanter N, Bentley SD, Robinson C, Maskell DJ, Parkhill J, Waller AS. 2009. Genomic evidence for the evolution of *Streptococcus equi*: host restriction, increased virulence, and genetic exchange with human pathogens. PLoS Pathogens, 5(3):e1000346.

Heidelberg JF, Seshadri R, Haveman SA, Hemme CL, Paulsen IT, Kolonay JF, Eisen JA, Ward N, Methe B, Brinkac LM, Daugherty SC, Deboy RT, Dodson RJ, Durkin AS, Madupu R, Nelson WC, Sullivan SA, Fouts D, Haft DH, Selengut J, Peterson JD, Davidsen TM, Zafar N, Zhou L, Radune D, Dimitrov G, Hance M, Tran K, Khouri H, Gill J, Utterback TR, Feldblyum TV, Wall JD, Voordouw G, Fraser CM. 2004. The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. Nature Biotechnology, 22(5):554-9.

Hotchkiss, R.D. 1948. The quantitative separation of purines, pyrimidines and nucleosides by paper chromatography. Journal of Biological Chemistry, 175: 315-332.

Ichida H, Matsuyama T, Abe T, Koba T. 2007. DNA adenine methylation changes dramatically during establishment of symbiosis. Federation of European Biochemical Societies Journal, 274(4):951-62.

Jeltsch A. 2002. Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. European Journal of Chemical Biology, 3(4):274-93.

Jeong H, Yim JH, Lee C, Choi SH, Park YK, Yoon SH, Hur CG, Kang HY, Kim D, Lee HH, Park KH, Park SH, Park HS, Lee HK, Oh TK, Kim JF. 2005. Genomic blueprint of *Hahella chejuensis*, a marine microbe producing an algicidal agent. Nucleic Acids Research, 33(22):7066-73.

Johnson EP, Mincer T, Schwab H, Burgin AB, Helinski DR. 1999. Plasmid RK2 ParB protein: purification and nuclease properties. Journal of Bacteriology, 181(19):6010-8.

Kapatral V, Ivanova N, Anderson I, Reznik G, Bhattacharyya A, Gardner WL, Mikhailova N, Lapidus A, Larsen N, D'Souza M, Walunas T, Haselkorn R, Overbeek R, Kyrpides N. 2003. Genome analysis of *F. nucleatum sub spp vincentii* and its comparison with the genome of *F. nucleatum* ATCC 25586. Genome Research, 13(6A):1180-9.

Klasson L, Walker T, Sebaihia M, Sanders MJ, Quail MA, Lord A, Sanders S, Earl J, O'Neill SL, Thomson N, Sinkins SP, Parkhill J. 2008. Genome evolution of *Wolbachia* strain wPip from the *Culex pipiens* group. Molecular Biology and Evolution, 25(9):1877-87.

Klasson L, Westberg J, Sapountzis P, Näslund K, Lutnaes Y, Darby AC, Veneti Z, Chen L, Braig HR, Garrett R, Bourtzis K, Andersson SG. 2009. The mosaic genome structure of the *Wolbachia* wRi strain infecting *Drosophila simulans*. Proceedings of the National Academy of Sciences USA, 106(14):5725-30

Klein R, Baranyi U, Rössler N, Greineder B, Scholz H, Witte A. 2002. *Natrialba magadii* virus phiCh1: first complete nucleotide sequence and functional organization of a virus infecting a haloalkaliphilic archaeon. Molecular Microbiology, 45(3):851-63.

Lim SK, Kim SJ, Cha SH, Oh YK, Rhee HJ, Kim MS, Lee JK. 2009. Complete genome sequence of *Rhodobacter sphaeroides* KD131J. Journal of Bacteriology, 191(3):1118-9.

Kobayashi I. 2001. Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution. Nucleic Acids Research, 29(18):3742-56.

Løbner-Olesen A, Skovgaard O, Marinus MG. 2005. Dam methylation: coordinating cellular processes. Current Opinion in Microbiology, 8(2):154-60.

Łobocka MB, Rose DJ, Plunkett G 3rd, Rusin M, Samojedny A, Lehnherr H, Yarmolinsky MB, Blattner FR. 2004 Genome of bacteriophage P1. Journal of Bacteriology, 186(21):7032-68.

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee JH, Díaz-Muñiz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'Sullivan D, Steele J, Unlu G, Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D. 2006. Comparative genomics of the lactic acid bacteria. Proceedings of the National Academy of Sciences USA, 103(42):15611-6

Malone T, Blumenthal RM, Cheng X. 1995. Structure-guided analysis reveals nine sequence motifs conserved among DNA amino-methyltransferases, and suggests a catalytic mechanism for these enzymes. Journal of Molecular Biology, 3;253(4):618-32.

Matsunaga T, Okamura Y, Fukuda Y, Wahyudi AT, Murase Y, Takeyama H. 2005. Complete genome sequence of the facultative anaerobic magnetotactic bacterium *Magnetospirillum sp.* strain AMB-1. DNA Research, 12(3):157-66.

Nallamsetty S, Waugh DS. 2007. A generic protocol for the expression and purification of recombinant proteins in Escherichia coli using a combinatorial His6-maltose binding protein fusion tag. Nature Protocols, 2(2):383-91.

Negri I, Franchini A, Gonella E, Daffonchio D, Mazzoglio PJ, Mandrioli M, Alma A. 2009. Unravelling the *Wolbachia* evolutionary role: the reprogramming of the host genomic imprinting. Proceedings of the Royal Society of London. Series B, 276(1666):2485-91.

Nelson KE, Fouts DE, Mongodin EF, Ravel J, DeBoy RT, Kolonay JF, Rasko DA, Angiuoli SV, Gill SR, Paulsen IT, Peterson J, White O, Nelson WC, Nierman W, Beanan MJ, Brinkac LM, Daugherty SC, Dodson RJ, Durkin AS, Madupu R, Haft DH, Selengut J, Van Aken S, Khouri H, Fedorova N, Forberger H, Tran B, Kathariou S, Wonderling LD, Uhlich GA, Bayles DO, Luchansky JB, Fraser CM. 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-

borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Research, 32(8):2386-95.

Paulsen IT, Banerjei L, Myers GS, Nelson KE, Seshadri R, Read TD, Fouts DE, Eisen JA, Gill SR, Heidelberg JF, Tettelin H, Dodson RJ, Umayam L, Brinkac L, Beanan M, Daugherty S, DeBoy RT, Durkin S, Kolonay J, Madupu R, Nelson W, Vamathevan J, Tran B, Upton J, Hansen T, Shetty J, Khouri H, Utterback T, Radune D, Ketchum KA, Dougherty BA, Fraser CM. 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science, 299(5615):2071-4.

Pieretti I, Royer M, Barbe V, Carrere S, Koebnik R, Cociancich S, Couloux A, Darrasse A, Gouzy J, Jacques MA, Lauber E, Manceau C, Mangenot S, Poussier S, Segurens B, Szurek B, Verdier V, Arlat M, Rott P. 2009. The complete genome sequence of *Xanthomonas albilineans* provides new insights into the reductive genome evolution of the xylem-limited Xanthomonadaceae. BMC Genomics, 10:616.

Poptsova MS, Gogarten JP. 2007. The power of phylogenetic approaches to detect horizontally transferred genes. BMC Evolutionary Biology, 21;7:45.

Puiu, D., Salzberg,S.L., Nene,V. and Lee,N. Direct Submission Submitted (13-NOV-2008) Center for Bioinformatics and Computational Biology, University of Maryland, 3115 Biomolecular Sciences Building #296, College Park, MD 20742, USA

Rasko DA, Ravel J, Økstad OA, Helgason E, Cer RZ, Jiang L, Shores KA, Fouts DE, Tourasse NJ, Angiuoli SV, Kolonay J, Nelson WC, Kolstø AB, Fraser CM, Read TD. 2004. The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. Nucleic Acids Research, 32(3):977-88.

Richter M, Kube M, Bazylinski DA, Lombardot T, Glöckner FO, Reinhardt R, Schüler D. 2007. Comparative genome analysis of four magnetotactic bacteria reveals a complex set of group-specific genes implicated in magnetosome iomineralization and function. Journal of Bacteriology, 189(13):4899-910.

Roberts RJ, Belfort M, Bestor T, Bhagwat AS, Bickle TA, Bitinaite J, Blumenthal RM, Degtyarev SKh, Dryden DT, Dybvig K, Firman K, Gromova ES, Gumport

RI, Halford SE, Hattman S, Heitman J, Hornby DP, Janulaitis A, Jeltsch A, Josephsen J, Kiss A, Klaenhammer TR, Kobayashi I, Kong H, Krüger DH, Lacks S, Marinus MG, Miyahara M, Morgan RD, Murray NE, Nagaraja V, Piekarowicz A, Pingoud A, Raleigh E, Rao DN, Reich N, Repin VE, Selker EU, Shaw PC, Stein DC, Stoddard BL, Szybalski W, Trautner TA, Van Etten JL, Vitor JM, Wilson GG, Xu SY. 2003. A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. Nucleic Acids Research, 31(7):1805-12.

Romero P, López R, García E. 2004. Genomic organization and molecular analysis of the inducible prophage EJ-1, a mosaic myovirus from an atypical pneumococcus.Virology, 322(2):239-52.

Salanoubat M, Genin S, Artiguenave F, Gouzy J, Mangenot S, Arlat M, Billault A, Brottier P, Camus JC, Cattolico L, Chandler M, Choisne N, Claudel-Renard C, Cunnac S, Demange N, Gaspin C, Lavie M, Moisan A, Robert C, Saurin W, Schiex T, Siguier P, Thébault P, Whalen M, Wincker P, Levy M, Weissenbach J, Boucher CA. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature, 415(6871):497-502.

Saridaki A, Sapountzis P, Harris HL, Batista PD, Biliske JA, Pavlikaki H, Oehler S, Savakis C, Braig HR, Bourtzis K. 2011. *Wolbachia* prophage DNA adenine methyltransferase genes in different *Drosophila-Wolbachia* associations. PLoS One, 6(5):e19708.

Sattley WM, Madigan MT, Swingley WD, Cheung PC, Clocksin KM, Conrad AL, Dejesa LC, Honchak BM, Jung DO, Karbach LE, Kurdoglu A, Lahiri S, Mastrian SD, Page LE, Taylor HL, Wang ZT, Raymond J, Chen M, Blankenship RE, Touchman JW. 2008. The genome of *Heliobacterium modesticaldum*, a phototrophic representative of the Firmicutes containing the simplest photosynthetic apparatus. Journal of Bacteriology, 190(13):4687-96.

Schübbe S, Williams TJ, Xie G, Kiss HE, Brettin TS, Martinez D, Ross CA, Schüler D, Cox BL, Nealson KH, Bazylinski DA. 2009. Complete genome sequence of the chemolithoautotrophic marine magnetotactic coccus strain MC-1. Applied and Environmental Microbiology, 75(14):4835-52

Seedorf,H., Fricke,W.F., Veith,B., Ji,F., Hagemeier,C., Liesegang,H., Strittmatter,A., Hinderberger,J., Brueggemann,H., Miethke,M., Thauer,R.K. and Gottschalk,G. Direct Submission Submitted (11-APR-2007) Institute of

Microbiology and Genetics, Goettingen Genomics Laboratory, Grisebachstrasse 8, Goettingen, Lower Saxony D-37077, Germany

Seshadri R, Adrian L, Fouts DE, Eisen JA, Phillippy AM, Methe BA, Ward NL, Nelson WC, Deboy RT, Khouri HM, Kolonay JF, Dodson RJ, Daugherty SC, Brinkac LM, Sullivan SA, Madupu R, Nelson KE, Kang KH, Impraim M, Tran K, Robinson JM, Forberger HA, Fraser CM, Zinder SH, Heidelberg JF. 2005. Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*. Science, 307(5706):105-8.

Skorupski K, Sauer B, Sternberg N. 1994. Faithful cleavage of the P1 packaging site (pac) requires two phage proteins, PacA and PacB, and two *Escherichia coli* proteins, IHF and HU. Journal of Molecular Biology, 243(2):268-82.

Shimodaira H, Hasegawa M. 2001.CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17:1246-1247.

Shimodaira H. 2002. An approximately unbiased test of phylogenetic tree selection. Systematic Biology, 51:492-508.

Shimodaira H, Hasegawa M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Molecular Biology and Evolution, 16:1114-1116.

Sobecky PA, Easter CL, Bear PD, Helinski DR. 1996. Characterization of the stable maintenance properties of the par region of broad-host-range plasmid RK2. Journal of Bacteriology, 178(7):2086-93.

Starkenburg SR, Chain PS, Sayavedra-Soto LA, Hauser L, Land ML, Larimer FW, Malfatti SA, Klotz MG, Bottomley PJ, Arp DJ, Hickey WJ. 2006. Genome sequence of the chemolithoautotrophic nitrite-oxidizing bacterium *Nitrobacter winogradskyi* Nb-255. Applied and Environmental Microbiology, 72(3):2050-63.

Starkenburg SR, Larimer FW, Stein LY, Klotz MG, Chain PS, Sayavedra-Soto LA, Poret-Peterson AT, Gentry ME, Arp DJ, Ward B, Bottomley PJ. 2008. Complete genome sequence of *Nitrobacter hamburgensis* X14 and comparative genomic analysis of species within the genus *Nitrobacter*. Applied and Environmental Microbiology, 74(9):2852-63.

Sternberg N, Coulby J. 1990. Cleavage of the bacteriophage P1 packaging site (pac) is regulated by adenine methylation. Proceedings of the National Academy of Sciences USA, 87(20):8070-4.

Sun S, Gao S, Kondabagil K, Xiang Y, Rossmann MG, Rao VB. 2011. The structure and function of the small terminase component of the DNA packaging machine in T4 like bacteriophages. Proceedings of the National Academy of Sciences USA, 109(3):817-22.

Takami H, Nakasone K, Takaki Y, Maeno G, Sasaki R, Masui N, Fuji F, Hirama C, Nakamura Y, Ogasawara N, Kuhara S, Horikoshi K. 2000. Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Research, 28(21):4317-31.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731-2739.

Tettelin H, Masignani V, Cieslewicz MJ, Donati C, Medini D, Ward NL, Angiuoli SV, Crabtree J, Jones AL, Durkin AS, Deboy RT, Davidsen TM, Mora M, Scarselli M, Margarit y Ros I, Peterson JD, Hauser CR, Sundaram JP, Nelson WC, Madupu R, Brinkac LM, Dodson RJ, Rosovitz MJ, Sullivan SA, Daugherty SC, Haft DH, Selengut J, Gwinn ML, Zhou L, Zafar N, Khouri H, Radune D, Dimitrov G, Watkins K, O'Connor KJ, Smith S, Utterback TR, White O, Rubens CE, Grandi G, Madoff LC, Kasper DL, Telford JL, Wessels MR, Rappuoli R, Fraser CM. 2005. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome". Proceedings of the National Academy of Sciences USA, 102(39):13950-5.

Valdes J, Quatrini R, Hallberg K, Dopson M, Valenzuela PD, Holmes DS. 2009. Draft genome sequence of the extremely acidophilic bacterium *Acidithiobacillus caldus* ATCC 51756 reveals metabolic versatility in the genus *Acidithiobacillus*. Journal of Bacteriology, 191(18):5877-8.

Volland S, Rachinger M, Strittmatter A, Daniel R, Gottschalk G, Meyer O. 2011. Complete genome sequences of the chemolithoautotrophic *Oligotropha carboxidovorans* strains OM4 and OM5. Journal of Bacteriology, 193(18):5043.

Vuilleumier S, Chistoserdova L, Lee MC, Bringel F, Lajus A, Zhou Y, Gourion B, Barbe V, Chang J, Cruveiller S, Dossat C, Gillett W, Gruffaz C, Haugen E, Hourcade E, Levy R, Mangenot S, Muller E, Nadalig T, Pagni M, Penny C, Peyraud R, Robinson DG, Roche D, Rouy Z, Saenampechek C, Salvignol G, Vallenet D, Wu Z, Marx CJ, Vorholt JA, Olson MV, Kaul R, Weissenbach J, Médigue C, Lidstrom ME. 2009. *Methylobacterium* genome sequences: a reference blueprint to investigate microbial metabolism of C1 compounds from natural and industrial sources. PLoS One, 4(5):e5584.

Wall T, Båth K, Britton RA, Jonsson H, Versalovic J, Roos S. 2007. The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall-altering esterase. Applied and Environmental Microbiology, 73(12):3924-35.

Ward N, Larsen Ø, Sakwa J, Bruseth L, Khouri H, Durkin AS, Dimitrov G, Jiang L, Scanlan D, Kang KH, Lewis M, Nelson KE, Methé B, Wu M, Heidelberg JF, Paulsen IT, Fouts D, Ravel J, Tettelin H, Ren Q, Read T, DeBoy RT, Seshadri R, Salzberg SL, Jensen HB, Birkeland NK, Nelson WC, Dodson RJ, Grindhaug SH, Holt I, Eidhammer I, Jonasen I, Vanaken S, Utterback T, Feldblyum TV, Fraser CM, Lillehaug JR, Eisen JA. 2004. Genomic insights into methanotrophy: the complete genome sequence of *Methylococcus capsulatus* (Bath). PLoS Biology, 2(10):e303.

Williams KP, Sobral BW, Dickerman AW. 2007. A robust species tree for the alphaproteobacteria. Journal of Bacteriology, 189(13):4578-86.

Wion D, Casadesús J. N6-methyl-adenine: an epigenetic signal for DNA-protein interactions. 2006. Nat Rev Microbiol.;4(3):183-92.

Wu CH, Huang H, Arminski L, Castro-Alvear J, Chen Y, Hu ZZ, Ledley RS, Lewis KC, Mewes HW, Orcutt BC, Suzek BE, Tsugita A, Vinayaka CR, Yeh LS, Zhang J, Barker WC. The Protein Information Resource: an integrated public resource of functional annotation of proteins. Nucleic Acids Research, 30(1):35-7.

Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadinejad N, Wiegand C, Madupu R, Beanan MJ, Brinkac LM, Daugherty SC, Durkin AS, Kolonay JF, Nelson WC, Mohamoud Y, Lee P, Berry K, Young MB, Utterback T, Weidman J, Nierman WC, Paulsen IT, Nelson KE, Tettelin H, O'Neill SL, Eisen JA. 2004. Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biology, 2(3):E69.

Yamada R, Iturbe-Ormaetxe I, Brownlie JC, O'Neill SL. 2011. Functional test of the influence of *Wolbachia* genes on cytoplasmic incompatibility expression in *Drosophila melanogaster*. Insect Molecular Biology, 20(1):75-85.

Ye YH, Woolfit M, Huttley GA, Rancès E, Caragata EP, Popovici J, O'Neill SL, McGraw EA. 2013. Infection with a Virulent Strain of *Wolbachia* Disrupts Genome Wide-Patterns of Cytosine Methylation in the Mosquito *Aedes aegypti*. PLoS One, 8(6):e66482.

5. Conclusions

In order to understand method of genetic exchange in endosymbiotic bacteria, this thesis examines the movement of *Wolbachia*, the bacteriophage WO, and one phage gene, the ParB-MTase, between hosts. *Wolbachia*, as well as other facultative endosymbionts, has the ability to move into new host populations, and to be horizontally transferred between phylogenetically distant insect species. This plasticity has led *Wolbachia* to become the most widespread heritable intracellular bacteria. The factors required for the establishment or maintenance of symbiosis between *Wolbachia* and its diverse hosts and the conditions required for colonization of a new insect species have not been identified despite intense study. The establishment of symbiosis represents a coordination of multiple genomes, including nuclear, bacterial and viral. The aim of this study was to examine the extent of horizontal transfer of *Wolbachia* and its WO prophage to investigate genetic mechanisms involved in these multitrophic exchanges.

Wolbachia strains infecting host-parasitoid systems were identified and typed in order to determine the movement of *Wolbachia* and WO prophages between two canola insect pests and their parasitoids. Phylogenetic analysis suggests that horizontal transfer has occured between *Plutella xylostella* and its parasitoid *Diadegma insulare*, but not between *Ceutorhycus obstrictus* and its parasitoid *Trictomalus lucidus*. Two different *Wolbachia* strains infect *Ceutorhycus obstrictus* (recently introduced to Alberta) and *Trictomalus lucidus* (currently undergoing host switching from native hosts). Examining *Wolbachia* density of *Ceutorhycus obstrictus* showed that the absence of horizontal transfer is not a

result of low *Wolbachia* titer but is more likely due to the recent establishment of this host-parasitoid system.

The genetic mechanism used by *Wolbachia* to infect a new host species is currently unknown. One of the major sources of genomic differences between *Wolbachia* strains is the presence of integrated prophages, WO. I have shown that all replicating WO bacteriophages share a conserved genomic backbone that includes modules for DNA packaging and head assembly, and tail morphogenesis. Although *Wolbachia* density is correlated with CI expression, no phage encoded virulence or symbiotic factors have been identified that may enhance *Wolbachia* transmission and facilitate colonization of new insect species.

One prophage encoded factor which has the potential to epigentically modify DNA is a methyltranferase containing a ParB-nuclease domain. The phylogenetic relationship of this gene revealed co-inheritance with *Wolbachia* in *Drosophila Wolbachia* strains. The phylogeny of this gene in prokaryotes showed that it is widespread and its position in each prokaryotic genome revealed a close association with bacteriophage DNA packaging genes. Unlike ParB-MTases in *Drosophila Wolbachia* strains, this gene has undergone horizontal transfer between distantly related bacterial species.

Deciphering molecular mechanisms, is essential for unraveling *Wolbachia's* ability to invade new hosts The results of my study provide the framework for future research into multitrophic interactions between insects, bacteria, and viruses in *Wolbachia*-host systems.

Appendix I: Phylogenetic Analysis

Sequence Alignment

Prior to analysis DNA or protein sequences were aligned using ClustalW using the IUB weight matrix. Retrieved sequence formats were either retrieved as either GenBank format (.seq), Fasta format (.fasta). Once the alignment is created it was the quality was examined using

Modeltest and Prottest

Using the nexus file for each DNA alignment, modeltest was run through PAUP* with the command "execute modelblock.txt". The outfile will contain the parameters for the model selected either from hierarchical likelihood ratio test (hLRTs) or Akaike information criterion (AIC). This can be implemented in PAUP* prior to maximum likelihood analysis.

Prottest was used for protein alignments using the graphical interface version. Alignments in nexus-sequence format were used with a BIONJ tree calculated and slow optimization strategy. Output will contain AIC or the Bayesian information criterion (BIC). These parameters for these models are implemented in PHYML.

PAUP - Parsimony Analysis*

Commands used for parsimony analysis.

log start=yes file=filename.txt;

tstatus;

cstatus;

set criterion=parsimony;

set increase=auto;

set rootmethod=mid;

set storebrlens=yes;

set storetreewts=yes;

bootstrap nreps=100 treefile=filename.tre search=heuristic/ addseq=random

nreps=10;

gettrees file=filename.tre storetreewts=yes;

savetree file=filename.tre savebootp=NodeLabels brlens=yes;

contree all/ strict=no majrule=yes percent=50 usetreewts=yes

file=finaltreename.tre;

end;

PAUP - Likelihood Analysis commands*

Similar to parsimony analysis with the following substitutions:

set criterion=like; instead of *set criterion=parsimony;*

Include Lset command to implement model parameters prior to boostrap analysis ,e.g.:

Lset Base=(0.34680 0.14410 0.23130) Nst=6 Rmat=(2.1114 5.3497 0.5505

1.2374 10.8359) Rates=gamma Shape= 0.2582 Pinvar=0.7612;

PHYML

Select the best protein substitution model determined by Prottest. Select bootstrap for branch support and the number of bootstraps to run. Enter values for proportion of invariable sites and gamma distribution parameter if values are known, otherwise keep as estimated. Enter number of substitution rate categories and select what values to optimize (e.g. tree topology, branch lengths, substitution rate). Lastly select topology search method (default is NNI).

MEGA

Choose construct/test maximum likelihood tree under phylogeny option. Select maximum likelihood as statistical method. For test of phylogeny select bootstrap method and enter the number of bootstraps to run. Select the best protein substitution model determined by Prottest, rates among sites and number of gamma categories. I selected complete deletion for gaps/missing data. Lastly select topology search method (default is NNI).