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*Wolbachia* are maternally transmitted endosymbionts that often alter their arthropod hosts’ biology to favor the success of infected females, and may also serve as a speciation microbe driving reproductive isolation. Two of these host manipulations include killing males outright or causing markedly reduced offspring survival when infected males mate with uninfected females, a phenomenon known as cytoplasmic incompatibility. Little is known about the mechanisms behind these phenotypes, but interestingly either effect can be caused by the same *Wolbachia* strain when infecting different hosts. For instance, *wRec* causes cytoplasmic incompatibility in its native host *Drosophila recens* and male killing in *D. subquinaria*. The discovery of prophage WO elements in most arthropod *Wolbachia* has generated the hypothesis that WO may encode genes involved in these reproductive manipulations. However, PCR screens for the WO minor capsid gene indicated that *wRec* lacks phage WO. Thus, *wRec* seemed to provide an example where phage WO is not needed for *Wolbachia*-induced reproductive manipulation. To enable investigation of the mechanism of phenotype switching in different host backgrounds, and to examine the unexpected absence of phage WO, we sequenced the genome of *wRec*. Analyses reveal that *wRec* diverged from *wMel* approximately 350,000 years ago, mainly by genome reduction in the phage regions. While it lost the minor capsid gene used in standard PCR screens for phage WO, it retained two phage WO regions encompassing 33 genes, several of which have previously been associated with reproductive parasitism. Thus, WO gene involvement in reproductive manipulation cannot be excluded and reliance on single gene PCR should not be used to rule out the presence of phage WO in *Wolbachia*. Additionally, the genome sequence for *wRec*
will enable transcriptomic and proteomic studies that may help elucidate the *Wolbachia* mechanisms of altered reproductive manipulations associated with host switching, perhaps among the 33 remaining phage genes.
Recent Genome Reduction of *Wolbachia* in *Drosophila recens* Targets Phage WO and Narrows Candidates for Reproductive Parasitism

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Abstract:
Wolbachia are maternally transmitted endosymbionts that often alter their arthropod hosts’ biology to favor the success of infected females, and may also serve as a speciation microbe driving reproductive isolation. Two of these host manipulations include killing males outright or causing markedly reduced offspring survival when infected males mate with uninfected females, a phenomenon known as cytoplasmic incompatibility. Little is known about the mechanisms behind these phenotypes, but interestingly either effect can be caused by the same Wolbachia strain when infecting different hosts. For instance, wRec causes cytoplasmic incompatibility in its native host Drosophila recens and male killing in D. subquinaria. The discovery of prophage WO elements in most arthropod Wolbachia has generated the hypothesis that WO may encode genes involved in these reproductive manipulations. However, PCR screens for the WO minor capsid gene indicated that wRec lacks phage WO. Thus, wRec seemed to provide an example where phage WO is not needed for Wolbachia-induced reproductive manipulation. To enable investigation of the mechanism of phenotype switching in different host backgrounds, and to examine the unexpected absence of phage WO, we sequenced the genome of wRec. Analyses reveal that wRec diverged from wMel approximately 350,000 years ago, mainly by genome reduction in the phage regions. While it lost the minor capsid gene used in standard PCR screens for phage WO, it retained two phage WO regions encompassing 33 genes, several of which have previously been associated with reproductive parasitism. Thus, WO gene involvement in reproductive manipulation cannot be excluded and reliance on single gene PCR should not be used to rule out the presence of phage WO in Wolbachia. Additionally, the genome sequence for wRec will enable transcriptomic and proteomic studies that may help elucidate the Wolbachia mechanisms of altered reproductive manipulations associated with host switching, perhaps among the 33 remaining phage genes.

Introduction:
Wolbachia are widespread obligate intracellular α-proteobacteria that infect around 40% of arthropod species (Zug and Hammerstein, 2012) and 47% of filarial nematodes (Ferri et al., 2011). These infection frequencies, if extrapolated to the diversity and abundance of their hosts, make Wolbachia perhaps the most widespread endosymbiont in animals. To maximize its propagation in arthropods, the maternally inherited Wolbachia has evolved an assortment of mechanisms to distort its host’s reproductive system in a manner that enhances the relative production of infected females. These mechanisms include feminization, parthenogenesis, male killing, and cytoplasmic incompatibility (CI), the most common phenotype and one that results in embryonic lethality when matings occur between infected males and uninfected females (Werren et al., 2008). Females harboring the same Wolbachia strain, meanwhile, can successfully mate and produce infected offspring with either infected or uninfected males, giving these females a selective advantage in populations of mixed infection status.

Interestingly, some Wolbachia strains are multipotent and can induce more than one type of reproductive manipulation depending on the arthropod host it infects (Fujii et al., 2001, Jaenike, 2007). In one striking example, the Wolbachia strain wRec causes CI in its native host, Drosophila recens, but when introgressed into a sibling species, D. subquinaria, it causes male killing (Jaenike, 2007). Moreover in a natural hybrid zone between these same two species, unidirectional CI plays a major role in reducing interbreeding and thus contributes to reproductive isolation between these species (Jaenike et al., 2006, Shoemaker et al., 1999). Even though the link between Wolbachia and CI has been known for over 40 years (Yen and Barr, 1971), the mechanisms by which Wolbachia accomplishes its reproductive manipulations remain unknown.

Despite the physical isolation resulting from its intracellular lifestyle, Wolbachia in arthropods are replete with mobile DNA (Wu et al., 2004) including a temperate bacteriophage named WO (Kent and Bordenstein, 2010, Metcalf and Bordenstein, 2012, Masui et al., 2000).
has been speculated that WO may be involved in *Wolbachia* reproductive manipulations due to the prevalence of ankyrin repeat genes in its genome ([Wu et al., 2004](#)), the pervasiveness of phage-encoded bacterial virulence factors ([Boyd, 2012](#)), and the frequent occurrence of phage WO in arthropod *Wolbachia* strains ([Gavotte et al., 2007](#)). However, evidence not supportive of this hypothesis includes the observations that CI is inconsistently associated with the presence of phage WO genes ([Sanogo et al., 2005](#), [Saridaki et al., 2011](#)) and that the penetrance of CI in *Nasonia* wasps is negatively correlated with densities of phage WO virions, as predicted by the phage density model ([Bordenstein et al., 2006](#), [Bordenstein and Bordenstein, 2011](#)). Interestingly, PCR screening for the WO minor capsid gene specified WO's absence in *wRec* ([Bordenstein and Wernegreen, 2004](#)), even though its closest relatives have large amounts of prophage DNA ([Wu et al., 2004](#), [Klasson et al., 2009](#)). Thus, the absence of phage WO in *wRec* would be a critical example of a *Wolbachia* strain causing multiple reproductive phenotypes but lacking WO.

To investigate the apparent lack of prophage WO genes and alternative genetic mechanisms behind *wRec*'s diverse phenotypic influences, we sequenced the *wRec* genome using next-generation sequencing technology with partial finishing via Sanger sequencing. We determined that although *wRec* lacks the WO minor capsid gene typically used in diagnostic screens, it does contain a number of prophage WO genes. Thus, the possibility that WO influences *Wolbachia* reproductive manipulations cannot be eliminated, and those WO genes present in *wRec* offer a streamlined candidate list of the WO genes that could cause reproductive parasitism. Additionally, the availability of genomic information for a *Wolbachia* strain that is known to switch reproductive phenotypes will enable genomic, transcriptomic, and proteomic approaches to investigate the mechanisms behind these phenotypes.

**Materials & Methods:**


The wRec genome sequencing reads and annotated contigs can be accessed from NCBI Bioproject PRJNA254527.

Wolbachia strain relatedness

Multi-locus sequence typing (MLST) genes were concatenated and a Bayesian phylogeny was inferred as previously described (Baldo et al., 2006). Briefly, selected fragments of MLST genes (coxA, gatB, fbpA, ftsZ, and hcpA) from Wolbachia strains with complete or nearly complete genome sequences were obtained from GenBank or the sequencing group’s online repositories, concatenated for a total length of 2,079bp, and aligned with MUSCLE (Edgar, 2004). jModelTest 2 (Darriba et al., 2012) was used to determine the best model of evolution for the set of MLST haplotypes (GTR + I + G), and a Bayesian phylogeny was inferred using Mr. Bayes (Ronquist et al., 2012) with a chain length of 1,100,000, burn-in of 100,000, and subsampling frequency of 200.

Genome sequencing and assembly

DNA was extracted from a pool of 10 female Wolbachia infected Pittsford strain D. recens flies using a Puregene DNA purification kit (Qiagen, Venlo, Limburg). Shotgun sequencing of the wRec genome was conducted using an Illumina Hi-Seq (Vanderbilt Sequencing Core, Nashville, TN) with 100 bp paired end sequencing. Reads were filtered using five available Wolbachia genomes, wBm (Foster et al., 2005), wMel (Wu et al., 2004), wRi (Klasson et al., 2009), wOo (Darby et al., 2012), and wPip (Klasson et al., 2008), by mapping reads to these genomes with length and similarity fractions of 0.5 and keeping all mapped reads, using CLC Genomics Workbench version 6.0.4 (CLC Inc, Aarhus, Denmark). A de novo assembly with a length fraction of 0.5 and similarity fraction of 0.8 was then performed on filtered reads. Sequencing of whole wRec-infected D. recens females produced over 24 million reads, of which
nearly 4% matched one or more previously sequenced Wolbachia genomes. De novo assembly of Wolbachia-filtered reads yielded 159 contigs. A de novo assembly of unfiltered reads was also performed and any contigs with a portion of its sequence matching contigs obtained from the filtered assembly were added to scaffolds in a search for novel genes. Separately, reads were mapped to the wMel genome with length and similarity fractions of 0.5 producing a rough consensus sequence to guide assembly of the de novo contigs into scaffolds, which were further refined with Sanger sequencing of PCR amplifications using primers designed to bind either end of putatively adjacent contigs to yield a final draft genome consisting of 43 scaffolds.

Annotation and comparative genomics

The wRec genome was annotated using MicroScope (Vallenet et al., 2009), supplemented with manual curation based on homology with wMel. A comparison of gene-gene identity between wMel and wRec was performed with a reciprocal best BLAST as previously described (Moreno-Hagelsieb and Latimer, 2008). For whole-genome alignments and analyses, wRec scaffolds were concatenated in the order in which the majority of their genes appear in wMel. Whole-genome alignment was performed with Mauve (Darling et al., 2010) and a circular genome plot was created with DNAPlotter (Carver et al., 2009). Manual annotations, BLAST searches, and sequence manipulation were performed with either CLC Genomics Workbench or Geneious V5.5.6 (Biomatters Ltd., Auckland, New Zealand). Ka/Ks rates and ratios were calculated using either single gene or concatenated whole genome CDS alignments with any alignments shorter than 30 amino acids removed (Buschiazzo et al., 2012), using the program DnaSP (Librado and Rozas, 2009). Genomic synteny was assessed with the Cloud Virtual Resource (CloVR) comparative pipeline (Angiuoli et al., 2011) and Sybil synteny gradient viewer (Riley et al., 2012) using the Data Intensive Academic Grid (DIAG) at the University of Maryland. The number of phage and phage-associated genes in Wolbachia genomes was
determined based on current GenBank annotations and includes genes in the phage-packaged
eukaryotic association module (Sarah Bordenstein, unpublished data).

Results:

Taxonomy of wRec

Phylogenetic analysis based on the concatenated multilocus sequence typing (MLST)
genomes (Baldo et al., 2006) confirms several previous reports that the supergroup A strain wRec is
closely related to wMel (Baldo et al., 2006, Werren et al., 1995, Ioannidis et al., 2007, Gueguen et
al., 2012), a widespread strain infecting D. melanogaster (Fig. 1). In addition, phylogenetic
analyses of each individual MLST gene support the same relationship of wMel as the closest
sequenced relative to wRec (data not shown). To date, all sequenced Wolbachia genomes in
supergroups A and B, including wMel (Wu et al., 2004), have contained significant amounts of
phage WO DNA. Thus the potential absence of WO in wRec was unexpected and precipitated the
genomic analysis described below.

Genome features of wRec with targeted reduction of prophage WO

Full sequencing statistics and an overview of wRec genome features are listed in Table 1.
wRec scaffolds (N=43) consisted of a total sequence length of 1,126,653 bp containing 1271
protein coding sequences. 99.7% of all nucleotides in coding sequences shared between wRec
and wMel were identical, indicating little divergence between these two closely related genomes
despite occupying hosts that diverged >50 million years ago (Ross et al., 2003). Based on a
previously established rate of synonymous substitution in Wolbachia of 0.9% per million years
(Raychoudhury et al., 2009), the genome-wide percentage of synonymous substitution (0.314%)
between wRec and wMel puts their divergence at approximately 350,000 years ago. There were
2,009 single nucleotide polymorphisms (SNPs) between shared coding genes in wMel and wRec,
and 599 (29.8%) of these SNPs were synonymous with an average $K_a/K_s$ ratio for each gene of 0.691. The vast majority of genes are highly conserved between $w$Rec and $w$Mel. More than 95% of orthologous gene pairs were 99% identical or greater and only ten gene pairs were less than 98% identical (Table 2). Most of these divergent genes code for hypothetical proteins and ankyrin repeat domain proteins. The $wsp$ surface antigen, a known hypervariable sequence in *Wolbachia* (Zhou et al., 1998), was also among the less conserved loci. All ten divergent genes contained insertions or deletions compared to $w$Mel in addition to one or more SNPs. Interestingly, four of these divergent genes, two coding for hypothetical proteins, an Ovarian Tumor (OTU)-like cysteine protease, and $wsp$, had $K_a/K_s$ ratios greater than one (Table 2), suggesting that they are evolving under positive selection, and the proteins they encode may be relevant to strain-specific host interactions. When these four genes were aligned to their homologs in $w$VitA, the closest relative of $w$Mel and $w$Rec, a roughly equal number of mutations in the OTU protease and $wsp$ genes in each strain matched the sequence in $w$VitA. However, for the two hypothetical proteins WREC_0649 (WD_0722) and WREC_1268 (WD_1278), the $w$Mel alleles matched $w$VitA in a majority of cases (18 out of 25 nucleotides and 49 out of 56 nucleotides, respectively), suggesting that the $w$Mel variants were ancestral and that these $w$Rec alleles experienced lineage-specific positive selection during *D. recens* infection.

Interestingly, there were only two $w$Rec genes without nucleotide homology to genes in $w$Mel, even when contigs from a de novo assembly of raw host/Wolbachia reads were mapped to scaffolds in a search for additional genes. These two genes, WREC_0318 and WREC_0319, are hypothetical proteins with >95% nucleotide identity to sequences in two other *Wolbachia* strains, $w$Ri and $w$Ha. Meanwhile, $w$Rec lacked any homologs of 43 $w$Mel genes (Table S1), all but one of which are phage-related (phage genes discussed below). The single non-phage gene without homology in $w$Rec is WD_0032, which codes for a hypothetical protein with 96% similarity to the C-terminus of an ankyrin repeat-containing siRNA binding protein in $w$Ri. As is the case for
many *Wolbachia* genomes, repetitive elements such as transposases and reverse transcriptases are abundant in *wRec* and have hampered closing of the genome. 77 such repetitive genes were found in *wRec*, and often appeared at the boundaries of scaffolds (Fig. 2). Although genomic rearrangement between the genomes cannot be completely assessed because the *wRec* genome is not closed, genes in *wRec* scaffolds were universally syntenic compared to *wMel* (Fig. 3), with the exception of a 5kb region containing WD_0042-WD_0051 (*WREC*_0853-*WREC*_0863), consisting of repetitive transposases, reverse transcriptases, hypothetical proteins, and pseudogenes. This region would have been located on the first *wRec* scaffold if syntenic, but instead is on scaffold 31 (Fig. 3).

**Prophage WO relics in the genome**

Whole-genome alignment of *wRec* and *wMel* revealed three major regions of genome reduction, with *wRec* lacking a large portion of both phage WO regions present in *wMel* as well as the entirety of the “Octomom” region (Chrostek et al., 2013) (Fig. 2), with only a bordering reverse transcriptase, *WREC*_0508 (*wMel* homolog WD_0506) present. Interestingly, although the minor capsid gene used in prior PCR surveys is absent, *wRec* does contain two major phage-related regions (Fig. 2). The first is a 19.2kb region (*WREC*_0261-*WREC*_0285) across three scaffolds that is homologous to 21 contiguous genes of *wMel* WO-A (WD_0276-WD_0296). This region in *wRec* is syntenic and 99.4% identical to its homologous region in *wMel*, with two exceptions. The *wRec* homolog (*WREC*_0270/*WREC*_0274) of WD_0285, an ankyrin repeat protein, is fragmented by the insertion of two reverse transcriptases and a gap in the scaffolds, and there is an 189bp insertion in *WREC*_0283 (WD_0294), another ankyrin repeat protein. If these two regions are included in the calculation of similarity, then the *wRec* WO-A phage region is 90.9% identical to the same region in *wMel*. The second *wRec* phage region contains 11.3kb and 7 genes (*WREC*_0559-*WREC*_0568) that are syntenic and homologous to part of WO-B in
**Discussion:**

*Divergence and genome reduction in wRec*

Genome analysis revealed that wMel and wRec are very closely related with an average of 99.7% nucleotide identity in coding regions shared by the two strains. We estimate that wRec and wMel diverged around 350,000 years ago. This estimate raises an interesting biogeographical question: how could *Wolbachia* have been transferred at this time between the widely allopatric Nearctic *D. recens* and Afrotropical *D. melanogaster*? Perhaps a widespread *Drosophila*-generalist parasitoid played a role in vectoring this endosymbiont between host species, as parasitoid wasps have been previously demonstrated as vectors for *Wolbachia* transfer ([Heath et al., 1999](http://example.com), [Vavre et al., 2009](http://example.com)). Molecular evidence suggests that the most recent *Wolbachia* sweep within *D. recens* occurred only 50,000 years ago, while *D. subquinaria* split from *D. recens* an estimated 600,000 years ago ([Shoemaker et al., 1999](http://example.com)). Thus, the divergence of wMel and wRec...
from their last common ancestor likely predated the most recent genetic sweep of *D. recens*, and

wRec infected *D. recens* after its incipient divergence from *D. subquinaria* (Werren and Jaenike, 1995, Shoemaker et al., 1999). Remarkably, these results suggest that wRec may have contributed
to reproductive isolation between these two species prior to the last glacial period 110,000 –
12,000 years ago, when their ranges are thought to have been allopatric (Jaenike et al., 2006).

However, we note caution in interpreting the estimated divergence times as variability in
mutation rates between bacterial lineages can skew the estimates.

The four wRec genes evolving under positive selection are of particular interest as they
may be potential mediators of Wolbachia-host interactions (Table 2). Indeed, wsp is known to be
involved in pathogenicity and host interaction (Uday and Puttaraju, 2012) while OTU-like
cysteine proteases have deubiquitinase activity facilitating the pathogenicity of intracellular
pathogens and viruses (Furtado et al., 2013, Makarova et al., 2000). Although the function of the
hypothetical proteins is unknown, the presence of transmembrane (TM) domains suggests
interaction with the bacterial membrane and potentially its *Drosophila* host. Additionally, it has
previously been speculated that the elevated rate of mitochondrial DNA evolution in *D. recens*
was due to hitchhiking in association with a series of positive selection events in its resident
Wolbachia (Shoemaker et al., 2004).

The major difference between the wMel and wRec genomes was the incipient genome
reduction of WO prophage regions. Remaining phage WO genes in wRec were often bordered or
interrupted by transposases, suggesting that transposase activity may have been involved in the
removal and degradation of major portions of WO genomes. Over 100kb of genetic material,
consisting mostly of phage-related genes, has likely been lost in wRec. Unlike the prophages
found in wMel (Wu et al., 2004), all of wRec’s WO regions lacked the head genes thought to be
necessary for mature virion formation (Metcalf and Bordenstein, 2012), including the orf7 minor
capsid protein used in previous PCR tests for WO (Bordenstein and Wernegreen, 2004). The lack
of such head genes suggests that wRec has lost the capacity to serve as a source of WO phage to infect other strains of Wolbachia. Future PCR screens may benefit from inclusion of more than one primer set, perhaps adding primers for a gene from the host adsorption module, which is highly conserved across WO prophages. However, it must be cautioned that the presence of multiple and variable degenerate WO haplotypes makes it impossible for any set of one or two primer pairs to detect all haplotypes.

Meanwhile, only 2,009 SNPs were present between the wMel and wRec genomes, indicating that gene deletion has been heavily favored over mutation. Such genome reduction is common in obligate intracellular bacteria, where many genes are expendable due to relaxed selection and there is limited contact with novel gene pools (Casadevall, 2008). Given the predatory nature of intact WO phages (Metcalf and Bordenstein, 2012, Bordenstein et al., 2006, Sanogo and Dobson, 2006), it may have been evolutionarily advantageous for wRec to eliminate the genes required for active phage production. It has been noted from TEM observations and quantitative studies that WO phage can lyse Wolbachia, resulting in an inverse correlation between bacterial and phage densities. Furthermore, because reproductive manipulations are dependent on a critical density of Wolbachia, high phage activity correlates with low expression of CI (Bordenstein et al., 2006). Since wRec exhibits high levels of CI in D. recens (Werren and Jaenike, 1995), while wMel shows lower levels of CI in D. melanogaster (Yamada et al., 2007), it is possible that wRec experiences a higher selective pressure to suppress phage, preserve high bacterial densities, and maintain compatibility with its host’s mating population. Thus, this interaction could be one possible explanation for the major loss of phage genes in wRec that are preserved in wMel.

Although Wolbachia has many more repetitive and mobile elements than most obligate intracellular bacteria (Bordenstein and Reznikoff, 2005) and frequently switches hosts on an evolutionary timescale (Vavre et al., 1999), it is worthwhile to note that there were only two
genes in wRec that were not present in wMel. It is possible that these genes were lost in wMel after divergence from its last common ancestor with wRec.

The phage WO hypothesis to explain reproductive parasitism

Because the Octomom region was completely absent in wRec, it is unclear whether wRec lost these genes after diverging from wMel, or whether the genes were acquired by wMel after divergence with their last common ancestor. Given that Octomom is not widespread in supergroup A Wolbachia, the latter possibility is likely. Moreover, although the function of Octomom in reproductive parasitism is unknown, it seems reasonable to conclude that the Octomom region is not needed for reproductive manipulations, as it is completely absent from wRec. Additionally, given the association of Octomom with increased Wolbachia virulence, proliferation, and host viral protection (Chrostek et al., 2013), we would predict that wRec would not possess these phenotypes, and may be a useful strain for confirming these associations.

It is intriguing that some WO genes are conserved in wRec while others were lost. One explanation for their preservation in wRec is that the remaining genes improve Wolbachia fitness. Indeed, prophage sequences code for advantageous virulence factors in a wide array of bacterial species (Brussow et al., 2004). Because previous PCR surveys suggested wRec did not possess phage WO, speculation that WO may be involved in Wolbachia reproductive manipulations has been largely disregarded (Bordenstein and Wernegreen, 2004). However, our sequencing shows that although the phage genomes are not complete, wRec contains many phage-related genes including some that could be involved in CI and/or male-killing. These include at least four ankyrin repeat proteins, whose repetitive domain has been long thought to facilitate Wolbachia-eukaryote interaction (Iturbe-Ormaetxe et al., 2005, Siozios et al., 2013). Additionally, several WO genes in wRec are homologs of genes recently implicated in CI. WREC_0560 is a transcriptional regulator with 88.3% identity at the amino acid level to wtrM in wPipMol, which
increases expression of an important regulator of meiosis in *Culex* mosquitos and is postulated to be a component of the molecular mechanisms of CI ([Pinto et al., 2013](#)). WREC_0566-WREC_0568 meanwhile, are homologous to WP_0282 and WP_0283, two genes in *wPip* that have been implicated in CI due to presence in the proteome of *Wolbachia*-infected, fertilized mosquito spermathecae, along with their pattern of presence/absence in CI and non-CI strains ([Beckmann and Fallon, 2013](#)). Although the *wRec* homolog of WP_0283 has been truncated by 427bp, it has 99.8% nucleotide identity to the gene in *wMel* (WD_0632) and an alternative reading frame enables the transcription of the remaining nucleotides in the same frame as the C-terminus of the homolog in *wMel*. Whether any of these WO genes are actually involved in *Wolbachia* host manipulations remains unclear, especially since it is unknown whether the remnants of phage WO are transcribed by *wRec*. However, the fact that these prophage regions are conserved suggests that they may have a role to play in the biology of *Wolbachia*.

### WO host adsorption genes

In addition to preservation of some potential reproductive manipulation mediators, prophage WO genes WREC_0263-WREC_0269 contain an intact host adsorption module that includes baseplate genes thought to be involved in the binding of WO to its bacterial host and insertion of phage DNA. Indeed, this host adsorption module is nearly universal in WO prophage, with very few degenerate phage haplotypes lacking these genes ([Kent et al., 2011](#)). Many intracellular bacteria, including *Wolbachia* ([Rances et al., 2008](#), [Pichon et al., 2009](#)), possess a type IV secretion system that secretes effectors into the host as a common strategy to subvert host-cell functions ([Voth et al., 2012](#)). A number of Gram-negative bacteria also possess a phage-like type VI secretion system ([Coulthurst, 2013](#)); these include several obligatory intracellular bacterial pathogens, such as *Anaplasma* and *Ehrlichia* ([Rikihisa and Lin, 2010](#)). Structural analyses have shown this type of secretion system bears a remarkable resemblance to the spike...
protein of phages (Silverman et al., 2012). Given these similarities, and the fact that the WO host adsorption module is almost universally present in sequenced arthropod Wolbachia (Kent et al., 2011), it is possible that Wolbachia may be using these genes to facilitate host-microbe interactions, as a way to inject CI factors, genes, or other host manipulation particles into its host.

Future Studies

The discovery of phage elements in wRec opens up new questions. Additional experiments will be needed to determine whether any conserved phage genes are involved in Wolbachia manipulations of its host biology. In addition, we have seen that using single gene markers of phage WO is not diagnostic of its absence. Thus, unsequenced Wolbachia strains that were thought to be phage-free by PCR assays need reevaluation. Another question that remains is how a single Wolbachia causes multipotent reproductive manipulations in different host backgrounds. The availability of genomic sequence for a multipotent Wolbachia strain will enable future transcriptomic and proteomic studies that could elucidate the genes involved in switching reproductive phenotypes.

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Table 1 (on next page)

*Rec sequencing and genome statistics*
|                       |               |
|-----------------------|---------------|
| Reads                 | 24,633,972    |
| wRec reads            | 955,730 (3.9%)|
| Contigs               | 159           |
| Scaffolds             | 43            |
| Average coverage      | 76.5          |
| Genome size           | >1,126,653 bp |
| GC content            | 35.2%         |
| CDS on scaffolds      | 1271          |
| Average CDS length    | 764 bp        |
| Average intergenic length | 130 bp     |
| Transfer RNA's        | 34            |
| Ribosomal RNA's       | 3 (23S, 16S, 5S) |
| Prophage regions      | 2             |
Table 2 (on next page)

_wMel_ genes with less than 98% nucleotide identity to their orthologs in _wRec_

Genes with a Ka/Ks ratio greater than one are highlighted.
| wMel Locus | wRec Locus | Function | Pairwise Identity (%) | wRec length | wMel length | # SNPs | # Non-synonymous SNPs | Ka/Ks Ratio | Other changes |
|------------|------------|----------|-----------------------|-------------|-------------|--------|----------------------|-------------|---------------|
| WD_0294    | WREC_028   | Ankyrin Repeat Domain Protein | 89.4 | 1815 | 1626 | 4 | 4 | - | 189 bp insertion |
| WD_0443    | WREC_044   | OTU-like Cysteine Protease | 97.1 | 927 | 906 | 7 | 6 | 1.59 | 21 bp insertion |
| WD_0550    | WREC_054   | Ankyrin Repeat Domain Protein *TM Domains | 87.4 | 789 | 990 | 2 | 2 | - | 99 bp deletion, C-terminal frameshift, alternate start/stop sites |
| WD_0722    | WREC_064   | Hypothetical Protein *TM Domains | 92.0 | 462 | 450 | 25 | 21 | 4.25 | 9 bp insertion, 3 bp insertion |
| WD_0996    | WREC_095   | Transposase | 89.1 | 744 | 801 | 1 | 0 | 0 | alternate start site, transposase insertion |
| WD_1007    | WREC_097   | Hypothetical Protein | 95.1 | 366 | 351 | 3 | 2 | 0.42 | 15 bp insertion |
| WD_1039    | WREC_100   | Collagen Triple Helix Repeat Protein | 97.5 | 405 | 1425 | 1 | 1 | - | 9 bp insertion, scaffold break |
| WD_1063    | WREC_103   | Wsp Surface Antigen | 97.9 | 708 | 714 | 9 | 8 | 2.55 | 6 bp deletion |
|    |    | Hypothetical Protein TM Domain |    |    |    |    |    |
|----|----|-------------------------------|----|----|----|----|----|
| WD_1278 | WREC_1268 | 92.1 | 2604 | 2766 | 56 | 51 | 6.07 |
| WD_1298 | WREC_1269 | 97.2 | 1974 | 1929 | 10 | 6  | 0.39 |

162 bp deletion
18 bp insertion, 27 bp insertion
Figure 1

WO phage is present in all sequenced supergroup A and B *Wolbachia* strains

A Bayesian phylogeny based on the concatenated *Wolbachia* multi-locus sequence typing genes is shown, consisting of selected strains with partial or full genome sequences and *w*Rec. All branches had posterior probabilities of 99% or greater. While all previously sequenced *Wolbachia* strains in group A and B possess phage WO elements, *w*Rec (indicated with arrow), was formerly thought to be phage-free.
Figure 2

**wRec genome comparison to wMel**

wRec scaffolds were concatenated in the order in which their genes appear in wMel to produce the circular genome above. Major regions of loss or gain compared to wMel are indicated outside the circle along with the number of genes involved. wRec genome features are indicated within the circle plot as follows (from outside-in): 1 (black): CDS in forward direction, and (magenta) genes not found in wMel; 2 (grey): CDS in reverse direction; 3 (red): scaffold break points; 4 (blue): WO regions; 5 (green): transposases and reverse transcriptases; 6 (purple/gold): GC content variation from average. WO prophage and related regions are shown and genes are categorized by color according to their likely functions and presence/absence in wRec. Locus tags for selected genes are indicated and dashed lines indicates breaks between scaffolds containing WO-A. The minor capsid gene of WO-A, which was used for prior PCR screens, is indicated with an asterisk.
Figure 3

Within-scaffold wRec synteny compared to wMel

wRec scaffolds were concatenated in the order in which they appear in wMel and within-scaffold synteny was analyzed. Genes are graphed as tick marks colored on a gradient from yellow to blue from left to right with wMel as the reference genome and each wRec gene colored according to the location of its homolog in the wMel genome. White spaces in wRec alone indicate the absence of homologous genes or genes with multiple paralogs whose synteny cannot be established, while white spaces shared by both genomes indicate intergenic regions. A 5kb region of rearrangement consisting of repetitive elements and hypothetical proteins is noted with an arrow, and phage-related regions are marked.
Figure 4

Number of phage genes in \( w \)Rec and its relatives

The total number of prophage, phage-associated, and WO-like island genes in each Wolbachia genome is plotted above a Bayesian phylogeny of their MLST genes. The approximate total length of phage genes in each genome is noted above each bar.
