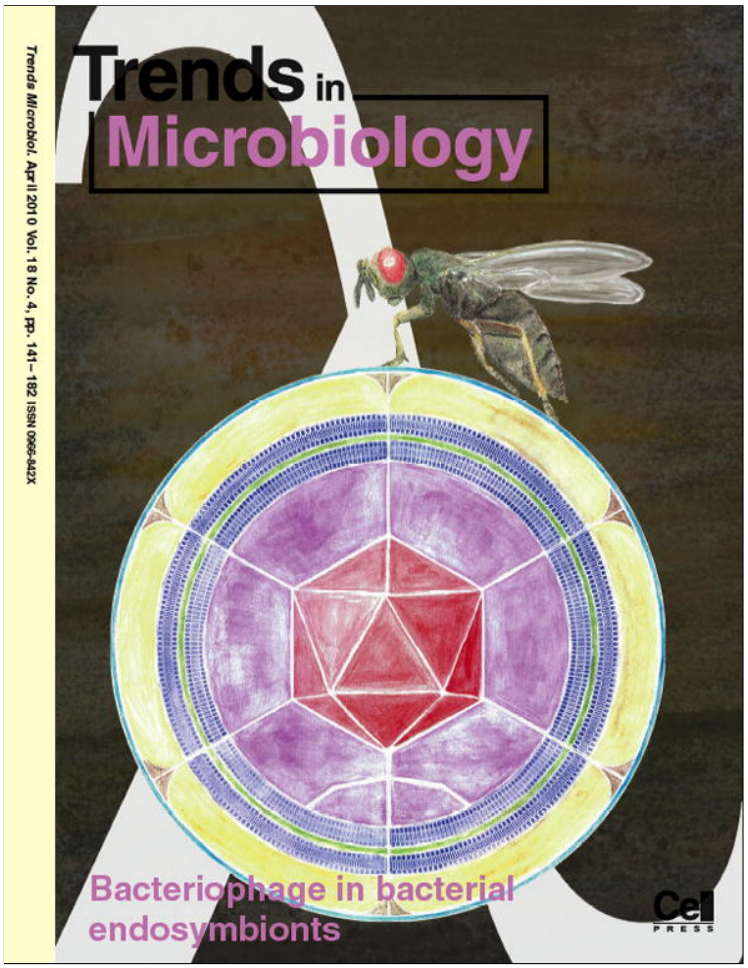

Review

Phage WO of *Wolbachia*: lambda of the endosymbiont world

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The discovery of an extraordinarily high level of mobile elements in the genome of *Wolbachia*, a widespread arthropod and nematode endosymbiont, suggests that this bacterium could be an excellent model for assessing the evolution and function of mobile DNA in specialized bacteria. In this paper, we discuss how studies on the temperate bacteriophage WO of *Wolbachia* have revealed unexpected levels of genomic flux and are challenging previously held views about the clonality of obligate intracellular bacteria. We also discuss the roles this phage might play in the *Wolbachia*-arthropod symbiosis and infer how this research can be translated to combating human diseases vectored by arthropods. We expect that this temperate phage will be a preeminent model system to understand phage genetics, evolution and ecology in obligate intracellular bacteria. In this sense, phage WO might be likened to phage λ of the endosymbiont world.

Mobile elements in intracellular bacteria

The restrictive lifestyle of obligate intracellular bacteria can lead to a near minimal genome state that encodes only essential functions. This reduction is associated with a genome-wide deletion bias, population bottlenecks and relaxed selection as a result of the ability of the bacterium to acquire nutrients from the host cell rather than synthesize them itself [1,2]. As a consequence of reductive evolution, mobile DNA elements have often been shown to be rare or absent from such streamlined bacteria [3–5]. However, genome sequence data shows that mobile elements are present at sometimes high frequency in obligate intracellular bacteria that switch hosts, including *Wolbachia*, *Rickettsia*, *Coxiella* and *Phytoplasma* [4,6–10]. Thus, past findings suggesting that streamlined bacterial genomes lack mobile DNA are being revisited with new hypotheses on how these elements invade and survive in these reduced genomes.

The tripartite arthropod–*Wolbachia*–phage WO system is emerging as a model to study the role of mobile elements in obligate intracellular bacteria. In the past few years, the publication of several complete WO sequences, the discoveries of rampant horizontal transmission between co-infections, and the tritrophic interactions between phage, *Wolbachia* and the arthropod host have propelled the field forward and will allow for rapid advancement in the study of WO evolution, function and activity.

The biology of bacteriophage WO

Wolbachia species are members of the obligate intracellular *Rickettsiales* and forge parasitic relationships with arthropods and mutualistic relationships, primarily with nematodes. During their 100-million-year association with their hosts, the maternally transmitted bacteria have evolved as "reproductive parasites" that cause cytoplasmic incompatibility (CI), feminization, parthenogenesis and male killing in arthropods, whereas in nematodes and some arthropods, they are mutualistic and can be required for host oogenesis or larval development [11–13]. In addition to modifying reproduction, *Wolbachia* spp. have recently been shown to confer resistance against RNA viruses [14–16], influence locomotion in response to food cues [17], and increase egg laying of females reared on low- or high-iron diets in *Drosophila* [18]. In *Asobara tabida* insects and cell lines from *Drosophila simulans* flies and *Aedes* mosquitoes, *Wolbachia* is involved in host iron metabolism [19]. These bacteria can also be transmitted horizontally across species, which has led to a pandemic-level distribution in invertebrates: current estimates suggest existence of *Wolbachia* in 66% of all arthropod species [20].

Identification of a bacteriophage in the *Wolbachia* infection of *Culex pipiens* mosquitoes was reported in the late 1970s [21], but confirmation of a *Wolbachia* phage did not occur until 20 years later, when a prophage region was identified in the genome of *Wolbachia* strain *w*Tai infecting *Teleogryllus taiwanemma* crickets [22]. Screening of *Wolbachia* infections from a variety of invertebrate hosts indicate that prophage WO (named after *Wolbachia*) is widespread in the genus [23–25]. PCR amplification of the minor capsid gene *orf7* showed that the phage infects 89% of the parasitic A and B *Wolbachia* supergroups (from arthropods) but is absent in the mutualistic C and D supergroups (from nematodes) [23,24]. However, vestiges

Glossary

Cytoplasmic incompatibility (CI): a sperm–egg incompatibility that renders embryos non-viable in crosses between infected males and uninfected females or females harboring a different strain of *Wolbachia*.
Feminization: the process by which infected male embryos are converted to morphological and functional females.
Haplotype: a distinct WO prophage type, based on nucleotide differences.
Male killing: the process by which male embryos or larvae are preferentially killed relative to female ones.
Parthenogenesis: a form of asexual reproduction in which infected females produce only female offspring.
Phage termini: the left and right terminal ends of the phage at which points the phage is integrated into the host genome. Upon excision of phage WO, these ends will join to form a circular phage genome.

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Table 1. Identification of WO prophages and active phage particles [a]

Insect		WO prophages			
Common name	Species	Particle size under TEM, nm	Prophages, *n*[b]	Identification of phage DNA from active particles?	References
Mosquito	*Culex pipiens*	20	5	NDA	[26,30]
	Aedes albopictus	~40	1	NDA	[31]
Cricket	*Teleogryllus taiwanemma*	40	1	PCR	[93]
Moth	*Cadra cautella*	40	2	PCR, cloning and sequencing	[28,29]
Wasp	*N. vitripennis*	~25	4	PCR	[27]
Fruit fly	*D. melanogaster*	~25	2	PCR	[25]
	D. simulans	ND	4	PCR	[25]

ND, not determined; NDA, no data available; TEM, transmission electron microscopy.
[a]Phage WO particles have been purified from several *Wolbachia*-insect systems using large-scale insect homogenization, density centrifugation and purification through filters. In some cases, DNA has been amplified from phage particle isolations, confirming the presence of active phage particles.
[b]Number of prophages in sequenced *Wolbachia* genomes.

of prophage DNA remain in the C and D supergroups, suggesting that at one point in evolutionary history they might also have harbored phage. Six prophage pseudogenes in the *w*Bm genome from the nematode *Brugia malayi* (Wbm5005, Wbm5030, Wbm5039, Wbm5040, Wbm5044, Wbm5080) are homologous to genes in *Wolbachia* A and B supergroups and in *Wolbachia*'s relatives *Rickettsia*, *Ehrlichia* and *Anaplasma*. These genes are not part of phage WO. One additional phage pseudogene, Wbm5055, is homologous to a conserved hypothetical protein gene found in WO prophages from *w*Pip (WP1304), *w*Kue (gp17) and *w*Ri (WRi_007190), and in non-WO regions in *w*Mel and other locations in *w*Ri. Distribution of WO in arthropods might be greater than the current estimates, as the primers used to screen for presence or *orf7* were not sufficiently degenerate to detect all of the *orf7* variants: for example, *Wolbachia* strain *w*Ri of *D. simulans* was initially reported as having a single WO haplotype [24], but the genome sequence confirmed four prophage copies in the genome, three of which are unique [8].

Icosahedral WO phage particles have been purified from several arthropods harboring *Wolbachia* infections (Table 1). The virion heads range in size from 20 to 40 nm and, occasionally, a tail structure has been identified by transmission electron microscopy (TEM) [26,27]. Although initially reported to be a linear double-stranded (ds)DNA molecule [28], the amplification of adjoined *att* sequences using inverse PCR demonstrated that the WO genome is circular [29] and replicates similarly to phage λ.

WO is a dynamic element that has a marked effect on the genetic diversity of *Wolbachia*. Complete sequences are known for prophages in strains *w*Mel [10], *w*Ri [8], *w*Kue [22], *w*CauB [28,29] and *w*Pip [30]. Analysis shows that WO molecular evolution is a complex process involving vertical transmission and horizontal transfer, recombination between phages, and hitchhiking of other mobile elements on WO. Although the details of phage transfer have not been discerned, evidence supports the potential movement of active WO particles between both related and divergent *Wolbachia* cells: electron microscopy shows particles aggregating outside of the *Wolbachia* cells after lysis and adjacent to developing spermatids in wasp testes [27]. Horizontal transfer of WO could occur either between several *Wolbachia* infections in a single host (Figure 1a) or, hypothetically, by paternal transmission of phage particles to a fertilized egg that harbors a phage-free *Wolbachia* strain (Figure 1b). PCR studies suggest that phage DNA might be transferred paternally from infected males to uninfected females (S.R. Bordenstein, unpublished data). WO horizontal gene transfer is strongly suggested by the fact that some divergent *Wolbachia* strains that co-infect the same host have identical *orf7* sequences [22,23,25,31]. This mechanism of transfer can be likened to an "intracellular arena" in which the host cell acts as a chemostat for phage transfers between *Wolbachia* co-infections [4,23]. Divergent phage haplotypes can also emerge from duplication of one initial viral infection. The two phage copies in *w*CauB are more similar to each other than to any other known WO sequence [29], and similarity for each of the five types in *w*Pip is highest between another WO prophage in the same genome [30]. However, it appears that some genes in the phage haplotypes inhabiting the same genome have been acquired from WO phages from other *Wolbachia* strains.

The flux of prophage WO genomes is also supported by intragenic recombination between different phage haplotypes. The nucleotide sequence of the minor capsid gene *orf7* from strain *w*KueA1 is chimeric, and population genetic analysis confirms the recombinogenic nature of WO [23]. The recombination rate of *orf7* is 12-fold greater than that of the rapidly evolving *Wolbachia* surface protein gene *wsp* and 15-fold greater than that of the WO terminase-encoding gene *orf2*. Why these phage genes (*orf7* and *orf2*), which are located less than 5 kb apart and required for lytic phage production, are recombining at different rates remains to be determined.

The contribution of prophage WO to *Wolbachia* genetic diversity is not limited to phage-associated genes. Insertion sequences (IS) are frequently found in WO genomes, including IS3, IS4, IS5, IS6, IS110 and IS630 family elements, [8,10,30] and might be a major factor driving phage recombination [32,33]. Genes encoding transposases are present in nearly all sequenced WO prophage genomes and can laterally transfer between *Wolbachia* strains [34]. If the presence of these IS elements on WO does not hinder its lytic ability, they could hitchhike within the phage genome as it spreads to new cells and move to new locations in the newly infected host genome [35]. IS elements are responsible for a significant amount of genetic diversity between many *Wolbachia* strains; for example, in *w*Pip, they truncate 44 genes [30].

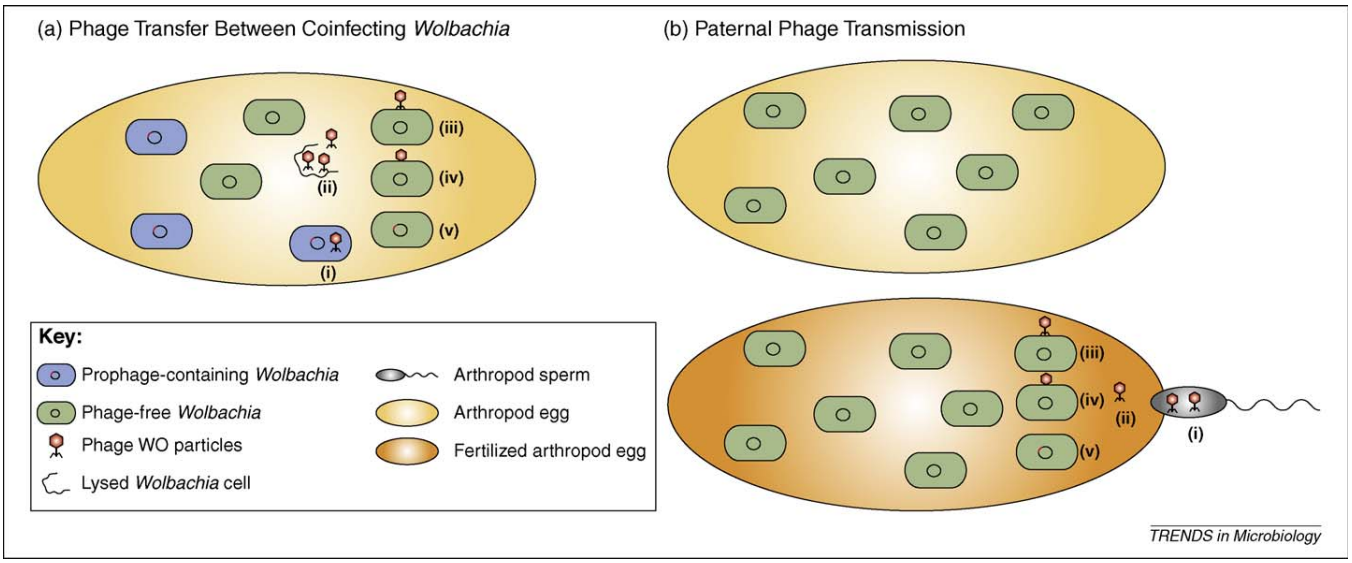

Figure 1. Horizontal transfer of bacteriophage WO. **(a)** Phage WO can transfer between two different *Wolbachia* strains that coinfect the same host cell [22,23,25,31]. The phage (i) becomes lytic and (ii) lyses its *Wolbachia* host cell. An active phage particle then (iii) attaches to a phage-free *Wolbachia* that co-infects the same host cell and injects its DNA (iv), at which point the DNA integrates into the chromosome (v). **(b)** Phage WO might also, hypothetically, be transmitted paternally by sperm from an infected male to the egg of a female carrying a phage-free *Wolbachia*. (i) Once the sperm fertilizes the egg, (ii) the transported phage is released and can infect *Wolbachia* as in steps iii–v above.

Evolution of the WO core genome

In dsDNA phages of bacteria with a free-living replicative stage, evolution is categorized by the Modular Theory [32,36,37]. According to this theory, a phage genome can be divided into functional units or modules (each one responsible for functions such as head or tail formation, lysis, lysogeny), which can be mixed by recombination with other phages. Each module often comprises genes that have a shared evolutionary history owing to their physical linkage and functional co-adaptation. Generalizing the principles of the Modular Theory to all dsDNA phages will require an expanded analysis in diverse ecological ranges [37]. In this regard, it seems opportune to test the theory on phages from obligate intracellular bacteria, as the intracellular niche might pose natural restraints on exposure to novel phage gene pools. Whereas extensive recombination would be expected in phages that are exposed to a multitude of other phages, recombination between unrelated phages that have a limited niche environment would confirm that the Modular Theory holds even in the obligate intracellular bacteria.

The identification of phage termini for two WO phages (WOCauB2 and WOCauB3) [29] and the complete sequences of 12 other active phages and prophages allow for an assessment of the WO core genome and an understanding of the molecular mechanisms by which these phages evolve. Modules for the assembly of head, baseplate and tail are readily identifiable based on gene homologies to the related lambdoid and P2 phages [10,22] (Figure 2), although tail module genes are present in only about half of the known WO sequences. Interspersed among the modules are genes of unknown function and others encoding putative virulence factors, transposases and ankyrin repeat (ANK) proteins [8,10,29,30,38]. It is likely that the conserved genes coding for hypothetical proteins located within specific modules are functionally required.

WO gene homologs occur in a diverse set of bacteria including Alpha-, Beta- and Gammaproteobacteria,

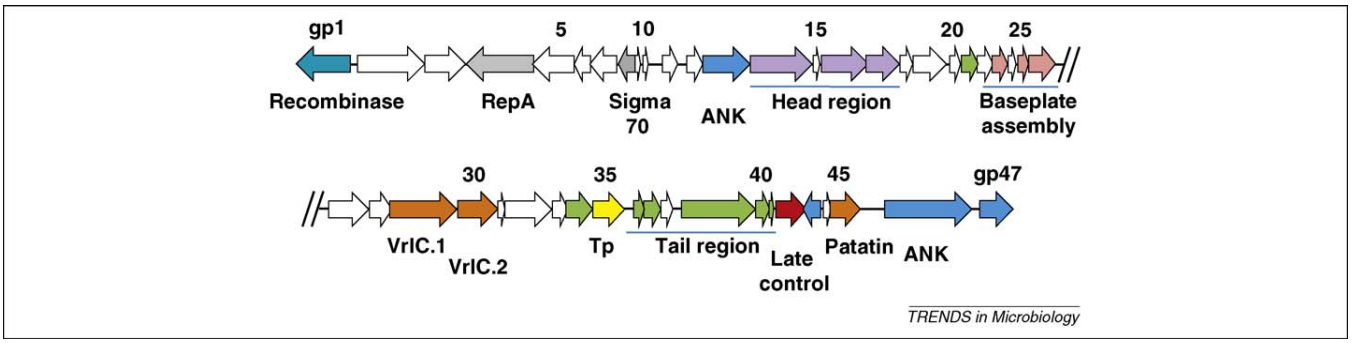

Figure 2. The genome architecture of phage WO. WOCauB2 from *w*CauB is an active phage based on the detection of excised intermediates by inverse PCR and genome sequencing [29]. Its genome is 43 kb in size and encodes 47 genes (numbered from gp1 to gp47). Functional gene homologs include a site-specific recombinase gene (teal), head region genes (purple), baseplate assembly genes (pink), tail protein genes (green) and a phage late control gene (red). Other interesting genes of note encode homologs of plasmid replication protein RepA and a sigma-70 transcription factor (grey). Several of the encoded proteins might interact with host proteins, including a patatin-like protein, VrlC.1 and VrlC.2 (orange) proteins, and ankyrin-repeat proteins (ANK, blue). Genes of unknown function are shown in white and transposase (Tp) is shown in yellow.

Table 2. Putative effectors and toxins encoded by the WO genomes of different *Wolbachia* strains

	Wolbachia strains								
Effector or toxin	**wCauB[a]**	**wRi[b]**	**wPip[c]**	**wMel[d]**	**wKue[e]**	**wWil[f]**	**wAna[g]**	**wMuni[h]**	**Putative function**
SpvB	B3gp45[i]								Interaction with host insect cells [29]
VrlA/VrlC.1	B1gp15, B2gp29, B3gp30	WRi_007010 pseudogene	WPa_1322, WPa_0440 pseudogene	WD0580		Wendoof_ 01000548	WwAna1071	WUni_ 000700	Secretion into host insect cells [28]
VrlC.2	B1gp16, B2gp30, B3gp31	WRi_007020	WPa_0441, WPa_1323	WD0579		Wendoof_ 01000385			Secretion into host insect cells [28]
Patatin	B2gp45, B3gp44	WRi_006880	WPa_1340, WPa_0272, WPa_0455	WD0565		Wendoof_ 01000457	WwAna0164		Entry into host cells
RhuM		WRi_005660, WRi_010320	WPa_0431	WD0259	gp8				Unknown
Addiction module toxin		WRi_005580	WPa_1330	WD0269, WD0600		Wendoof_ 01000378	WwAna0127	WUni_ 002995	Killing of cells lacking WO
DNA methylase		WRi_005640, WRi_010300	WPa_0258, WPa_0317, WPa_0429, WPa_1310	WD0263, WD0594		Wendoof_ 01000687, Wendoof_ 01000839	WwAna0008		Methylation of host DNA, protection of phage against restriction enzymes

[a]Phages WOCauB1 (Accession No. AB161975.2), WOCauB2 (AB478515.1) and WOCauB3 (AB478516.1) from *w*CauB of *Cadra cautella* [28,29].
[b]Prophages from the *w*Ri genome of *D. simulans* Riverside (NC_012416) [8].
[c]Prophages from the *w*Pip genome of *C. quinquefasciatus* Pel (NC_010981) [30].
[d]Prophages from the *w*Mel genome of *D. melanogaster* (AE17196) [10].
[e]Prophage WO from *w*Kue of *Ephestia kuehniella* (AB036666.1) [22].
[f]Prophage homologs from *w*Wil of *Drosophila willistoni* (NZ_AAQP00000000).
[g]Prophage homologs from *w*Ana of *Drosophila ananassae* (NZ_AAGB00000000).
[h]Prophage homologs from *w*Muni of *Muscidifurax uniraptor* (NZ_ACFP00000000).
[i]All gene numbers refer to the primary annotation of each genome in GenBank.

Firmicutes, Cyanobacteria and Bacteriodetes, which could indicate that these phage genes have been transferred between a number of phyla [29]. Evidence that WO can acquire genes from or transmit genes to bacteria other than *Wolbachia* includes the 70% nucleotide identity between prophage genes in *w*Mel and a segment of a *Rickettsial* plasmid [38]. WO homologs also occur in the prophage regions of the facultative intracellular parasite *Bartonella henselae* [39]. Current data indicates that whereas phage WO genomes are modular, the functional gene modules do not readily exchange DNA with unrelated phages, as WO genes often have the highest sequence similarity to genes in other WO phages. The rarity of modular exchange with unrelated phages is probably a result of the unique intracellular niche that phage WO occupies. Instead, evolutionary forces such as point mutation, deletions, recombination and inversions tend to be the dominant modes of diversification. Notably, these modes of phage diversification still cause some of the largest fractions of absent or divergent genes between closely related *Wolbachia* genomes [38].

Lifecycle of phage WO

Although the lytic and lysogenic nature of temperate phage WO has been demonstrated, the genetic mechanisms that drive prophage induction and lytic activity are currently unknown. Phage particles have been visualized for several *Wolbachia* strains (Table 1) but the precise identification of phage termini has occurred only from phages of the *Wolbachia* infection of *Cadra cautella* moths [29]. A serine recombinase gene homolog that is probably responsible for integration of these phages is located at the termini of phages WOCauB2 (Figure 2) and WOCauB3, but the exact binding sites of the recombinase are unknown. These WO phages are not flanked by an inverted repeat, and comparison of the joined ends of the active phage genome (*attP*) with the integrated prophage terminal sequences (*attR* and *attL*) and flanking *Wolbachia* sequences (*attB*) found in common only a single nucleotide T in WOCauB2 and a trinucleotide TTG in WOCauB3 [29]. The serine recombinase gene found in the WOCauB phages is different to that of other sequenced WO prophages, with the exception of one *w*Ri phage (WRi_012450), indicating that the other WO phages use a different site-specific recombination mechanism or are degenerate.

Expression of phage genes can be sex-specific and age-specific relative to the host arthropod. The expression of the minor capsid protein gene *orf7* was compared between developmental stages and sexes in *Wolbachia* from three different populations of *C. pipiens* and one from *Culex quinquefasciatus* [26]. Adult females from all four *Culex* strains expressed *orf7*, whereas adult males of only two strains did the same. Expression also varied between developmental stages. In all four populations, eggs and early larval stages expressed *orf7*, but expression in later-stage larvae varied in the populations from no expression to strong expression. Strong expression returned by the pupal stage in three of the four strains. This evidence suggests that the biology of phage WO is closely linked to that of the arthropod host, hypothetically through direct interaction between host-encoded proteins and proteins encoded on the phage (ANK proteins, for example) or through insect-induced changes in *Wolbachia* that have a downstream affect on phage WO. Studies using *Nasonia* parasitoid wasps have recently found that lytic phage production appears to be influenced by several abiotic and biotic factors including

insect age, host species background and temperature (S.R. Bordenstein, unpublished data).

Effectors, toxins and the tripartite relationship of WO, *Wolbachia* and arthropods

In addition to the genomics and transmissibility of bacteriophage WO, there is considerable interest in the phage's function in the *Wolbachia*–arthropod symbiosis. Mobile elements were initially hypothesized to be responsible for causing reproductive parasitism because they can promiscuously transfer new functions between strains, potentially explaining the phylogenetic curiosity that different types of reproductive parasitism (CI, male killing, feminization, parthenogenesis) do not cluster in groups in the *Wolbachia* tree. Analysis of WO genome sequences has identified several candidate proteins for interaction with eukaryotic cells (Table 2). Genes having homology to *vrlA* and *vrlC* (virulence-associated genes [40] on a pathogenicity island of the sheep pathogen *Dichelobacter nodosus*) are located in WO prophage sequences [28]. In *D. nodosus,* VrlC contains a conserved motif typically associated with sialidases [40]. A sialidase that cleaves glycoconjugates on cell surfaces might be involved in pathogenesis or *Wolbachia*'s ability to scavenge nutrients from the host cell as seen in a diverse range of bacteria such as *Pasteurellaceae* [41] and some *Mycoplasma* [42,43]. Notably, a weak correlation between sequence variability of VrlC and CI was observed in *C. pipiens* mosquitoes [44].

Seven WO prophages also contain a homolog of a *Rickettsia* gene coding for a patatin-like phospholipase belonging to the phospholipase A_2 family. Proteins containing patatin-like domains have been linked to virulence in *Legionella pneumophila* [45] and *Pseudomonas aeruginosa* [46–48]. It is possible that the WO homolog might assist *Wolbachia* entry into host cells or be involved in other arthropod host cell interactions [29]. Further, the *w*Ri, *w*Mel and *w*Pip prophages include a gene that might be part of a toxin/antitoxin system, by functioning as an 'addiction' module (i.e. killing those cells lacking the mobile element [49]). Homologs of this gene are also found elsewhere within the *Wolbachia* genome.

WOCauB3 contains a gene encoding a protein similar to the *Salmonella enterica* ADP-ribosylating toxin, SpvB [50–52]. The WO homolog shows some similarity to bacterial proteins of the RHS and YD-repeat families, which have been implicated in interactions with eukaryotic host cells [53,54]. A protein with similar motifs is present in APSE, the bacteriophage of *Hamiltonella defensa* that confers pea aphid resistance to parasitoid attack [55,56]. Additionally, the SpvB homolog has sequence homology to a family of insecticidal toxins [29]. The diversity of putative effector proteins and toxins encoded between prophage genomes suggests that WO is involved in many important facets of *Wolbachia* biology. How the phage's effectors and toxins affect its host will be an important future topic of study.

Whereas the identity of virulence factors varies between phage copies, genes coding for ankyrin-repeat (ANK) proteins are found in large number in *Wolbachia* genomes and particularly in the vicinity of or encoded on WO [8,10,30,57]. ANKs are protein motifs that can mediate protein–protein interactions, act as transcription factors, and modify the activity of cell-cycle regulatory proteins in eukaryotes [58–60]. ANK proteins often contain transmembrane domains or signal peptides, offering two different mechanisms by which they can interact with the host cells: surface expression or secretion into the insect cell. Because eukaryotic ANK proteins span functions involved in a variety of cell processes, *Wolbachia* ANK proteins have been hypothesized to have a role in reproductive parasitism.

The expression of ANK proteins was compared between *w*Mel, which causes CI in *D. melanogaster* and the closely-related *w*Au, which infects *D. simulans* but does not cause CI [61]. It was found that one ANK gene is absent in *w*Au but present in *w*Mel, and the sequence of seven genes encoding ANK proteins in *w*Au differed significantly from that of the *w*Mel homologs. Of these seven genes, one showed a difference in expression as a result of a gene truncation caused by an insertion sequence within the open reading frame. Two of these ANK genes are located in the prophage region of *w*Mel [62]. These differences in ANK protein expression and structure are candidates for the inability of *w*Au to cause sexual alteration. In *Culex* mosquitoes, two ANK genes associated with a prophage region are expressed sex-specifically and have sequence divergence associated with CI [63]. However, other genes such as *orf7* show sex-specific expression even though there is no association of sequence variability with CI [26]. Although it is tempting to speculate that WO ANK genes could be involved in *Wolbachia*–host interactions, current evidence for this is complex and insufficient.

The Phage Density Model

Phage sequence analyses find no phylogenetic clustering of WO genotypes between the four major *Wolbachia*-induced

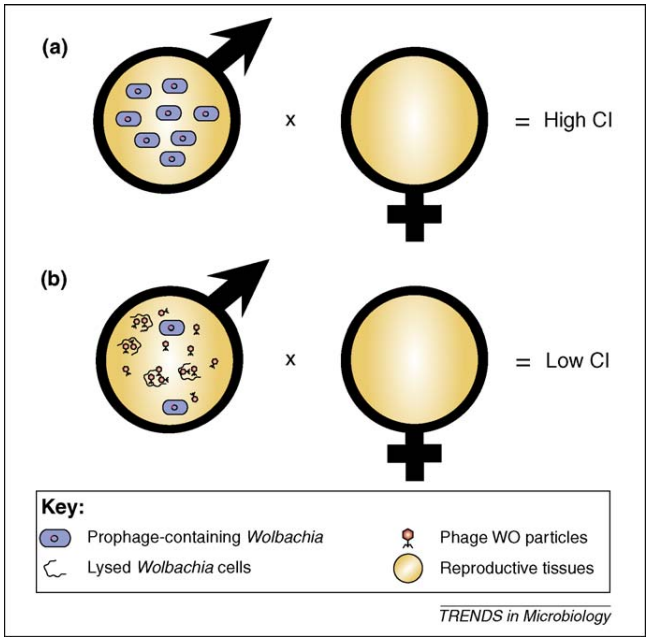

Figure 3. The phage density model of cytoplasmic incompatibility (CI). **(a)** When phage WO is lysogenic and titers of *Wolbachia* are high in male reproductive tissues, high levels of CI prevent the production of viable offspring after mating with an uninfected female. **(b)** When phage WO in these *Wolbachia* becomes lytic, *Wolbachia* cell titers decrease as a result of cell lysis and cause the infected male and uninfected female to produce an increased number of offspring [27].

sexual alterations. Further, there are some *Wolbachia* strains that induce CI, male killing or parthenogenesis but lack the WO prophage [23,24]. These facts led to the development of the Phage Density Model as an alternative, but not mutually exclusive, explanation for the role of phage WO in sexual alterations (Figure 3) [27]. This theory proposes that variations in phage lysis are linked to the expression of sexual alterations through variations in *Wolbachia* densities. According to the model, lytic phages kill *Wolbachia* cells and thereby reduce bacterial densities in the tissues associated with reproductive modification. Because bacterial density is one of the most crucial determinants of expression of *Wolbachia* functions [64–67], its variation affects the expression of sexual alterations. Evidence from TEM observations and quantitative studies of phage–*Wolbachia* interactions in *Nasonia vitripennis* wasps show particles exiting lysed cells into host reproductive tissues, and an inverse association of phage and *Wolbachia* titers [27]. A supergroup-B strain of *N. vitripennis* with a mean phage density of <2 (estimated as the relative number of copies of the WO *orf7* gene in relation to those of *Wolbachia* gene *groEL*) exhibited 100% CI, whereas a supergroup-A strain with a mean phage density of 6 exhibited a decreased level of 67.7% CI. Among the supergroup-A infected males that showed variation in CI levels, males with complete CI had significantly lower phage densities than males expressing incomplete CI.

The model and evidence emphasize that the first tenet of phage function is that phages are parasites of bacteria, and that clarifying the separate roles of lytic and lysogenic phage development in *Wolbachia* biology will effectively structure inquiries into the function of phage WO. Currently, the weight of the experimental evidence suggests that the lytic phage is a mobile genetic parasite that can reduce *Wolbachia* densities, whereas the role of the lysogenic prophage remains a topic of future interest. Further, not all systems will harbor phage WO or show the same patterns as that observed in *N. vitripennis*. For instance, the phage density model does not appear to be supported in *Culex* mosquitoes [68], but sample sizes in this study were too small to exclude it.

Biomedical applications for phage WO

Wolbachia has become increasingly important to human health and disease through two routes. First, vector control programs aimed at curbing the spread of insect-vectored diseases such as malaria and dengue fever will rely on the ability to release insect vectors transfected with *Wolbachia* infections that reduce the vectorial competence [69–74]. Second, the discoveries that river blindness, lymphatic filariasis [75] and heartworm [76] are associated with *Wolbachia*-induced pathologies raised the likelihood that antimicrobial therapies targeting *Wolbachia* might be effective in treatment of the systems [76–79]. For insect diseases, current strategies include (i) using *Wolbachia* to carry a transgene that would inhibit spread of the infectious agent [80,81], (ii) infecting mosquitoes with a life-shortening strain of *Wolbachia* so that the insect vector dies before it is capable of transmitting disease [71,82–84], or (iii) releasing *Wolbachia*-infected males into the wild so that CI will inhibit mosquito reproduction and cause the native populations to crash to less-threatening levels [85,86]. In order for the first strategy to be effective, population replacement of natural vectors with transgenically modified ones must be rapid and overcome fitness costs associated with the transgene [81]. The speed with which *Wolbachia* can spread through a population as a result of its effect on host reproduction makes it an ideal method to transmit a transgene in wild populations of insects [81,87–89].

The ability for *Wolbachia* to be used in biological control of diseases is dependent on successful infection of mosquito vectors. In the first steps towards showing that a mosquito line for population replacement could be generated, the dengue vector *Aedes aegypti* was successfully infected with life-shortening *Wolbachia* strain *w*MelPop [73,90]. Under laboratory conditions, the lifespan of *A. aegypti* was halved after infection with *w*MelPop [71], and in older mosquitoes, a reduction in the ability to blood feed was noted [91], suggesting that a release of *w*MelPop into wild populations of mosquitoes could significantly reduce dengue transmission.

Strategies to combat malaria have shown less promise. Although it was possible to infect cell lines of the malaria vector *Anopheles gambiae* with *w*MelPop [92], the infection was avirulent [70]. In theory, mosquitoes infected with a transgenic *Wolbachia* expressing a protein that could hinder the transmission of malaria to humans would be an effective way to decrease the spread of the disease. Unfortunately, there are currently no tools available for the genetic manipulation of *Wolbachia* and no reports of successful transformation. Phage WO particles could be developed into the first transgenic tool of *Wolbachia*, as active phages might succeed in vectoring transgenes to recipient *Wolbachia* cells where traditional transformation strategies have failed. Additionally, the integrase and *att* sites in WO could be used to construct vector systems capable of integrating into the *Wolbachia* genome [29]. Once integration of mini-WO constructs is successful, phage WO could also be used as a knockout insertion element so that candidate genes underlying *Wolbachia* traits such as reproductive parasitism could be rapidly identified.

Concluding remarks and future directions

Studies of phage WO have shown its potential to have a substantial effect on the symbiosis between *Wolbachia* and host arthropods. Extrapolation of the phage infection frequency places WO in potentially millions of insect species, where it can contribute to *Wolbachia* genomic diversity and function in a number of ways, including horizontal gene transfer between different *Wolbachia* and between other endosymbionts, exchange of other mobile elements such as insertion sequences, intragenic recombination, gene loss and an alteration in *Wolbachia* densities that can affect the penetrance of reproductive parasitism. The phenotypic effects on the eukaryotic host cell could be equally diverse. WO encodes several different classes of proteins, such as virulence factor homologs and ANK proteins, which could influence *Wolbachia* or the arthropod host. Additionally, lytic WO decreases reproductive parasitism in *Nasonia* by

Box 1. Questions for future research

Evolution:

- Does the lack of modular exchange typify phage WO genome evolution?
- How common is phage WO across the *Wolbachia* genus?

Ecology:

- What are the common mechanisms by which phage particles transfer?
- Do phage WO sequences cluster with geography or host range?
- What genetic factors regulate the temperate lifecycle of phage WO?

Host interaction:

- Is WO retained because of a benefit to the *Wolbachia* bacterium or to the host arthropod?
- How applicable is the phage density model to other *Wolbachia*–arthropod systems?
- What role do the encoded effector proteins play in interactions with bacterial and eukaryotic cells?

Applications:

- Can WO be used as a transgenic vector system either through active phage particles or through a mini-integration construct?

lowering *Wolbachia* densities, but whether this relationship holds in other *Wolbachia*–arthropod relationships remains to be determined. Beyond *Wolbachia* lysis, the precise mechanisms by which the phage interacts with the invertebrate host is a topic of interest.

There is hope that phage WO particles might overcome current barriers to transform *Wolbachia* or that functional aspects of WO integration could be used to generate vector systems capable of supplying transgenes into *Wolbachia* genomes. Successful transformation of *Wolbachia* must first be demonstrated before a vector system would be of use.

Study of the biology of WO would not only expand knowledge of phage evolution and function, but could also lead to a role of this phage in the treatment of insect-vectored diseases. Therefore, future research on phage WO should encompass a wide variety of themes, including endosymbiosis, phage biology and arthropod vector control (Box 1). As the study of new phages in diverse ecological niches necessitates new models akin to the well-studied phage λ, phage WO seems an adequate model for obligate intracellular bacteria.

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