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The Complexity of Virus Systems: The Case of Endosymbionts

Jason A. Metcalf¹ and Seth R. Bordenstein^{1,2,*}

¹Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA

²Department of Pathology, Microbiology, and Immunology, Vanderbilt University, Nashville, TN, USA

Abstract

Host-microbe symbioses involving bacterial endosymbionts comprise some of the most intimate and long-lasting interactions on the planet. While restricted gene flow might be expected due to their intracellular lifestyle, many endosymbionts, especially those that switch hosts, are rampant with mobile DNA and bacteriophages. One endosymbiont, *Wolbachia pipientis*, infects a vast number of arthropod and nematode species and often has a significant portion of its genome dedicated to prophage sequences of a virus called WO. This phage has challenged fundamental theories of bacteriophage and endosymbiont evolution, namely the phage Modular Theory and bacterial genome stability in obligate intracellular species. WO has also opened up exciting windows into the tripartite interactions between viruses, bacteria, and eukaryotes.

Introduction

Bacterial endosymbionts that replicate within eukaryotic cells are extremely widespread in nature. In addition to the endosymbiont-derived organelles of mitochondria and chloroplasts, more recently-evolved bacterial endosymbionts are abundant in nature, occurring in virtually all eukaryotic hosts [1]. Historically, obligate intracellular endosymbionts were thought to be devoid of mobile and laterally acquired DNA given their isolated niche, but recent studies have shown that the ecology of bacterial endosymbionts significantly influences the amount of their genome populated by mobile elements such as phages (Fig. 1) [2,3]. Here, we discuss the prevalence of endosymbiont viruses and focus on recent reports describing the evolution, host interactions, and scientific applications of one of the most widespread and well-studied endosymbiont viruses, phage WO.

Prevalence of phages in endosymbionts

Bacteriophages are the most abundant biological entity on Earth, outnumbering their unicellular hosts by at least an order of magnitude [4]. Although free-living bacteria are less restrictive targets for phages, the most recent survey of mobile genetic elements in bacteria has shown that many endosymbionts possess equal amounts of mobile DNA including phages [2]. While endosymbionts that are strictly vertically transmitted from mother to offspring, such as *Buchnera*, *Wigglesworthia*, and *Blochmannia*, often lack phages, the genomes of those that switch hosts, such as *Chlamydia*, *Rickettsia*, *Phytoplasma*, and

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*Corresponding author: Seth Bordenstein, Vanderbilt University, VU Station B, Box 35-1634, Nashville, TN 37235, s.bordenstein@vanderbilt.edu, phone: 1.615.322.9087, fax: 1.615.343.6707.

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Wolbachia, often contain a high percentage of mobile DNA [3]. Indeed, 21% of the genome of the *wPip* strain of *Wolbachia pipientis* is comprised of mobile DNA, including five prophages [5], and phages are present in *Chlamydia pneumoniae* isolates throughout the globe [6]. Additionally, endosymbionts not currently infected by phages often show evidence of past infections. For example, *wBm*, the *Wolbachia* strain infecting the nematode *Brugia malayi*, has at least six phage pseudogenes even though it currently lacks a whole prophage [7,8]. Even mitochondria, which have been obligate endosymbionts for over a billion years, possess genes that likely were derived from ancient bacteriophages [9].

The phages of *Wolbachia* in particular merit closer examination for several reasons: (1) *Wolbachia* is likely the most widespread endosymbiotic genus on the planet, infecting an estimated 66% of all arthropod species [10] as well as most medically and agriculturally important nematodes [11]. (2) Many *Wolbachia* strains are rampantly infected with a group of temperate dsDNA bacteriophages named WO [7,12]. (3) *Wolbachia* exhibit numerous influences on their hosts that ensure their spread as reproductive parasites [13] (see section below on reproductive parasitism), and WO may play a role in these effects [14]. (4) WO phages have several potential applications as tools for understanding endosymbiont evolution and manipulating their biology.

Evolution of WO

The availability of a large number of sequenced WO phages and *Wolbachia* genomes has enabled a close examination of WO genome structure and evolution [15]. There are five strains of *Wolbachia* in which active phage particle production has been demonstrated [12,16–18], each of which contains prophages with complete head, baseplate, and tail gene modules essential for proper phage function (Fig. 2). Interestingly, *Wolbachia* strains that harbor a complete WO phage usually have additional WO prophages that are degenerate, transcriptionally inactive [19], and, with a few exceptions [5,20], not closely related to other prophages in the same strain [15].

It is commonly understood that dsDNA bacteriophages evolve mainly through frequent horizontal gene transfer of contiguous sets of unrelated genes with a similar function (i.e. tail genes, head genes, lysis genes, etc) between phages in a common gene pool. This tenet is termed the Modular Theory [21]. However, analysis of 16 WO sequences revealed for the first time that, although WO phages are modular, they do not evolve according to the Modular Theory but rather through point mutation, intragenic recombination, deletion, and purifying selection (Fig. 3) [15]. Thus, although WO is prevalent in *Wolbachia*, its obligate intracellular niche limits the exposure of WO to other phages with which to recombine. Indeed, all evolutionarily recent horizontal transfer events among WO phages are between co-infections of intracellular bacteria in the same eukaryotic host, reflecting the fact that endosymbionts have relatively little interaction with free-living bacteria or their phages (Fig. 3). Examples of these transfers include a 52 kb phage transfer between *Wolbachia* strains *wVitA* and *wVitB* coinfecting the parasitic wasp *Nasonia vitripennis* [22], and multiple phage transfers between coinfecting *Wolbachia* strains in natural populations of the leaf beetle *Neochlamisus bebbianae* [23]. Transfer can also occur between different species of obligate or facultative intracellular bacteria, such as between *Wolbachia* and a plasmid from a *Rickettsia* endosymbiont of the tick *Ixodes scapularis* (Fig. 4) [24].

In addition to transfer of phages between bacteria, lateral gene transfer of *Wolbachia* genes into their eukaryotic hosts' genomes is surprisingly common, with *Wolbachia* genes found in at least seven insect species and four nematode species [25–28]. These inserts range in size from less than 500bp in *Nasonia* to nearly the entire *Wolbachia* genome in *Drosophila ananassae* [25]. Interestingly, these transfers often include WO prophage regions [25] or

sequences adjacent to WO in the *Wolbachia* genome (Fig. 4) [26]. Given the extensive host range of these endosymbionts, many more as yet undiscovered horizontal transfer events are likely.

Involvement of WO in reproductive parasitism

Perhaps the most tantalizing concept in the study of WO is the idea that WO may influence the biology of not only *Wolbachia*, but also *Wolbachia*'s arthropod hosts. *Wolbachia* have evolved several mechanisms for manipulating their hosts' reproduction to ensure their spread and maintenance in a population by increasing the evolutionary fitness of *Wolbachia*-transmitting females [13]. These mechanisms include (1) male killing (male offspring die during embryogenesis), (2) feminization (genetic males develop into fertile females), (3) parthenogenesis (virgin females produce all female broods) and (4) cytoplasmic incompatibility (CI), an asymmetrical crossing incompatibility in which offspring of *Wolbachia*-infected males and uninfected females die during early embryogenesis. The idea that WO could be involved in these manipulations is based on the precedent that bacteriophages commonly encode virulence factors and other genes promoting the fitness of both phage and its host [29]. Even endosymbiont phages may provide such a function. For example, APSE, a phage of *Hamiltonella defensa*, defends *H. defensa*'s host, the aphid *Aphidius ervi*, against parasitic wasps, likely through a phage-encoded toxin of unknown mechanism [30,31]. Additionally, *Wolbachia* genomes and especially WO prophage regions are replete with ankyrin-repeat proteins [32], a motif known to mediate diverse protein-protein interactions in eukaryotes [33]; thus they could facilitate *Wolbachia*'s reproductive manipulation of its hosts.

Wolbachia-induced reproductive manipulations are remarkably complex. For example, bidirectional CI blocks the production of offspring between two insects harboring different strains of *Wolbachia* in some cases but not others [34], leading to several theories for how CI functions. The Lock and Key Model postulates that numerous combinations of modification (mod) factors alter arthropod sperm such that they cannot develop in uninfected eggs, while rescue (resc) factors repair this defect if the egg is infected with a compatible strain of *Wolbachia* [34,35]. Another theory, the Goalkeeper Model, posits that only two factors exist, but that their concentration or activity level accounts for incompatibility between some strains [36]. In any case, these intricate CI patterns have enabled a search for correlations between strain compatibility and WO, although the results have been somewhat contradictory [12,18,37,38].

One hypothesis is that a WO DNA methyltransferase gene may encode the mod and/or resc factors of CI [37]. This theory fits well with the fact that sperm DNA appears to be modified in the hosts of mod+ *Wolbachia* strains and that DNA methylation is altered during feminization of the leafhopper species *Zyginidia pullula* when infected with *Wolbachia*, although methylation patterns have not yet been investigated in CI [39]. Remarkably, all resc+ group A *Wolbachia* examined have a WO-encoded *met2* methyltransferase gene, whereas resc- *Wolbachia* do not. However, this correlation does not extend to group B *Wolbachia*, suggesting that if *met2* is the resc factor in group A, it is not universal or its equivalent in group B has not yet been recognized [37]. The *met2* gene has been constitutively expressed in *Drosophila melanogaster* and was unable to cause or rescue CI in wMel-infected flies [40]. Nevertheless, there are several additional genes found in mod+, resc+ strains but not mod-, resc- strains [32], so it remains possible that the WO methyltransferase is involved in CI but requires additional proteins. Examination of transcription of WO genes has shown differential expression of haplotypes of a capsid gene, *orf7*, between sexes, strains, and life stages of *Culex pipiens* mosquitoes [18]; however, there has been no obvious correlation between *orf7* haplotypes and CI patterns in several

species [12,38]. Perhaps most damning to the hypothesis that WO underlies reproductive parasitism is the fact that some *Wolbachia* strains without WO still manipulate host reproduction [12]. Therefore, if WO genes are directly involved in arthropod reproductive manipulation, it is likely not universal in all strains, but could be part of a larger interplay with other *Wolbachia* genes and host factors.

Even if WO genes are not directly involved in reproductive manipulations, there is significant evidence that WO indirectly influences CI by controlling *Wolbachia* densities in the host, a theory termed the Phage Density Model [7]. In *wVitA*, which infects *Nasonia vitripennis* and contains active, lytic WO, densities of *Wolbachia* and WO are inversely related, as are *Wolbachia* densities and CI severity [16]. Interestingly, altering *Wolbachia* environmental factors does not abolish this three-way interaction. Introgression to move the *wVitA* strain from its native host into a related species of wasp, *N. giraulti* increased *Wolbachia* load, decreased WO densities, and increased CI [41], while rearing insects at temperature extremes had the opposite effect [42]. In *wPip*-infected *Culex pipiens* mosquitoes under conditions where WO is not lytic, this correlation is not seen [43]. These results strongly suggest that lytic WO influences CI by altering *Wolbachia* densities. Additionally, this interaction is influenced by host factors in a tripartite relationship between WO, *Wolbachia*, and their insect host.

Applications of WO

One of the greatest limitations in *Wolbachia* research is the inability to successfully transform these bacteria. Until the *Wolbachia* genome can be manipulated, it is unlikely that fundamental questions regarding the mechanism of CI and other aspects of *Wolbachia* biology will be definitively answered. Fortunately, WO offers hope as an avenue for accomplishing this genetic manipulation. Recombinases and attachment sites for WO integration have been identified that could be exploited to this end [44], although there is significant diversity in recombinases and no integration site common to all WO prophages [15]. The large size of the WO genome, diversity of phage sequences, and intracellular lifestyle of *Wolbachia* are all obstacles to overcome, but development of a WO DNA-delivery vector would be a colossal advance in the study of *Wolbachia*.

WO also has a potential therapeutic application. Although *Wolbachia* is a reproductive parasite in most arthropods, in many parasitic nematodes, including those causing filariasis and river blindness in humans, *Wolbachia* is mutualistic and required for the nematodes' reproduction [13]. Indeed, elimination of *Wolbachia* with antibiotic therapies has been successful in treating filarial diseases [45]. WO may encode useful gene products for inhibiting *Wolbachia*, as phages often express numerous proteins for manipulation and inhibition of their hosts. Potential candidates in WO include lysozymes, which lyse bacterial cell walls [46], and patatins, which have a phospholipase activity [47]. Lysozymes have been identified in two WO phages, while patatins are nearly universal in WO [15]. An understanding of how WO manipulates and lyses *Wolbachia* may enable development of small molecules with similar functions, or the use of WO's own proteins as therapeutics if they can be accompanied by an appropriate delivery system [48].

Conclusions

Given the abundance and range of *Wolbachia* and its phage WO, a firm grasp of the biology in this system will be important for understanding endosymbiont viruses in general and their interactions with their hosts. WO has already tested fundamental questions in evolutionary theory and hinted at fascinating host interactions at multiple levels of symbiotic

relationships. Further study of WO and perhaps use of WO as a tool for genetic manipulation will no doubt lead to even more intriguing discoveries in the future.

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Highlights

- Viruses are abundant in many endosymbionts despite their intracellular niche
- WO, a phage of *Wolbachia*, is one of the most widespread endosymbiont viruses
- The Modular Theory of phage genome evolution does not apply to WO
- WO forms a tripartite symbiotic relationship between arthropods, bacteria, and viruses
- WO has potential as a vector for *Wolbachia* transformation and in therapeutics

Figure 1.

Effects of microbial ecology on exposure to phage gene pools. Facultative intracellular bacteria have the largest exposure to bacteriophage genes due to their flexible lifestyle involving both the free-living and intracellular environments; thus, they have the greatest amount of mobile DNA in their genomes. Extracellular bacteria have an intermediate amount of mobile DNA, while obligate intracellular bacteria have the least. However, intracellular bacteria that switch hosts and can be horizontally transmitted often retain a large quantity of mobile DNA including phages.

Figure 2.

WO particle and genome structure. (A) Typical appearance of a tailed bacteriophage, color-coded by structural groups. (B) Electron micrograph of WO particles. Examples of phage particles are indicated with arrowheads. Shown is WO isolated from μ CauB in the moth *Ephestia kuehniella*. Photo courtesy of Sarah Bordenstein. (C) The modular genome of prophage WO. Relative portions of the genome dedicated to individual modules and the modules' orientation and arrangement are shown for lysogenic WOCauB2. Other WO strains have modules in differing arrangements and orientations and some may lack various modules all together. Not all genes are shown.

Phage in obligate intracellular bacteria

Phage in free-living bacteria

Figure 3.

Evolution of bacteriophages in endosymbionts and free-living bacteria. Bacteriophages (1) of endosymbionts (2) are restricted in their interactions with other phages due to the barrier of the eukaryotic host membrane (3). Their genomes evolve mainly through recombination (4), point mutation (5), and deletion (6). Bacteriophages (7) of free-living bacteria (8) can more freely interact with each other facilitating modular gene exchange (9) and forming viruses consisting of parts of each parent strain (10). Thus, free-living but not endosymbiont phages evolve by the Modular Theory.

Figure 4.

Examples of gene flow between WO, *Wolbachia*, and insects. WO prophage sequences (1) have been transferred between coinfections of different *Wolbachia* strains (2 and 3) on several occasions. Additionally, *Wolbachia* genes have been transferred to a *Rickettsia* plasmid (4), and both WO and *Wolbachia* genes have been found in multiple insect host genomes (5).