Bacteriophage Flux in Endosymbionts (*Wolbachia*): Infection Frequency, Lateral Transfer, and Recombination Rates

Seth R. Bordenstein and Jennifer J. Wernegreen

The Marine Biological Laboratory, Josephine Bay Paul Center for Comparative Molecular Biology and Evolution, Woods Hole, Massachusetts

The highly specialized genomes of bacterial endosymbionts typically lack one of the major contributors of genomic flux in the free-living microbial world—bacteriophages. This study yields three results that show bacteriophages have, to the contrary, been influential in the genome evolution of the most prevalent bacterial endosymbiont of invertebrates, *Wolbachia*. First, we show that bacteriophage WO is more widespread in *Wolbachia* than previously recognized, occurring in at least 89% (35/39) of the sampled genomes. Second, we show through several phylogenetic approaches that bacteriophage WO underwent recent lateral transfers between *Wolbachia* bacteria that coinfect host cells in the dipteran *Drosophila simulans* and the hymenopteran *Nasonia vitripennis*. These two cases, along with a previous report in the lepidopteran *Ephestia cautella*, support a general mechanism for genetic exchange in endosymbionts—the "intracellular arena" hypothesis—in which genetic material moves horizontally between bacteria that coinfect the same intracellular environment. Third, we show recombination in this bacteriophage; in the region encoding a putative capsid protein, the recombination rate is faster than that of any known recombining genes in the endosymbiont genome. The combination of these three lines of genetic evidence indicates that this bacteriophage is a widespread source of genomic instability in the intracellular bacterium *Wolbachia* and potentially the invertebrate host. More generally, it is the first bacteriophage implicated in frequent lateral transfer between the genomes of bacterial endosymbionts. Gene transfer by bacteriophages could drive significant evolutionary change in the genomes of intracellular bacteria that are typically considered highly stable and prone to genomic degradation.

Introduction

Bacteriophages are among the most abundant group of replicating entities on the planet (Bergh et al. 1989; Hendrix et al. 1999) and constitute a significant force in bacterial genome evolution. At least 62% (51/82) of surveyed bacterial genomes harbor prophage elements that can in some cases occupy up to 20% of a host genome (Casjens 2003). Bacteriophages are also notable catalysts of lateral transfer and can shuttle large portions of unique DNA into recipient host genomes (Ohnishi, Kurokawa, and Hayashi 2001; Banks, Beres, and Musser 2002; Ventura et al. 2003). For example, intraspecific genome comparisons between two *Escherichia coli* isolates reveal that prophage genes account for nearly half of the strain-specific sequences in one of the isolates (Ohnishi, Kurokawa, and Hayashi 2001). In addition, bacteriophages are one of the most important transporters of virulence genes in free-living bacteria and are known to encode extracellular toxins, invasion proteins, and adhesion factors for their bacterial hosts (Miao and Miller 1999; Boyd, Davis, and Hochhut 2001; Boyd and Brussow 2002).

Given the growing interest in bacteriophages and bacterial genome evolution, it is notable that prophage elements are largely missing from bacteria with highly reduced genome sizes. Of the remaining 38% (31/82) of genomes that lack prophage elements, many are smaller than 2 Mb (Casjens 2003). A growing number of bacterial endosymbiont genomes fall into this class, and that may come with little surprise. It is conventionally thought that the highly specialized genomes of obligate endosymbiotic bacteria get refuge from mobile parasitic elements because they are sheltered by their intracellular lifestyle and lack several genes involved in recombination pathways. Indeed, the published genomes of primary endosymbiotic bacteria of insects, such as *Buchnera* (Tamas et al. 2002), *Wigglesworthia* (Akman et al. 2002), and *Blochmannia* (Gil et al. 2003) are void of selfish elements or their remnants. These highly stable bacterial genomes form obligate mutualistic relationships with their insect hosts, are typically specialized within a limited host range, and experience extraordinary genome stability (Tamas et al. 2002).

However, the discoveries of bacteriophages in two unrelated bacterial endosymbiont systems, the γ-Proteobacteria secondary symbiont of aphids (van der Wilk et al. 1999; Sandstrom et al. 2001) and the widespread α-Proteobacteria of arthropods, *Wolbachia* (Masui et al. 2000; Masui et al. 2001; Wu et al. 2004), have raised new questions about whether bacteriophages can play important roles in the evolution of some endosymbiont genomes. Both endosymbionts harbor bacteriophages that are over 20 kb in length and, in the case of bacteriophage WO from *Wolbachia*, regions have the highest amino acid similarity to genes of the temperate bacteriophages λ and P2 of *E. coli* (Masui et al. 2000). Determining whether these bacteriophages are active and important contributors to genomic diversification in their endosymbiont hosts remains an important area of research for these elements. Studies of bacteriophage WO gene expression and particle enrichment support the activity and lytic ability of these phages (Masui et al. 2000; Masui et al. 2001). For endosymbiotic bacteria that have an intracellular lifestyle and, therefore, reduced opportunities for genetic exchange, active bacteriophages could constitute both a serious threat and central source of evolutionary innovation, even more so than in free-living bacteria.

Interest in the bacteriophages of *Wolbachia* is particularly heightened by the evolutionary success and plasticity of its endosymbiont host. *Wolbachia* are one of

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E-mail: sbordenstein@mbl.edu.

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the most abundant intracellular bacteria in the biosphere, occurring in 17% to 75% of all insect species, as well as mites, isopods, thrips, and filarial nematodes (Werren, Windsor, and Guo 1995; Jeyaprakash and Hoy 2000; Bourtzis and Miller 2003; Charlat, Hurst, and Mercot 2003). Estimates, therefore, place *Wolbachia* in millions of invertebrate species; if WO is as abundant as its *Wolbachia* host, then WO could be one of the most copious lineages of bacteriophages in invertebrate cells.

Apart from its remarkable distribution, *Wolbachia* show unusually high levels of genomic and phenotypic plasticity (Bandi, Slatko, and O'Neill 1999; Wu et al. 2004) that present the basis for its division into at least six major supergroups of *Wolbachia* (labeled A to F). Supergroups A to F diverged from each other approximately 100 MYA (Lo et al. 2002), and during the radiation, they established different lifestyles. The A and B *Wolbachia* of arthropods largely became reproductive parasites (that induce feminization, parthenogenesis, male killing, or cytoplasmic incompatibility), whereas the C and D *Wolbachia* forged mutualistic relationships with their nematode hosts. The mutualistic lineages tend to fit the traditional wisdom of endosymbiont genome evolution—they are reduced in size (0.9–1.1 Mb) (Sun et al. 2001), experience strict vertical inheritance, and do not show evidence of recombination (Jiggins 2002). In contrast, the parasitic genomes are significantly larger (1.3–1.6 Mb) (Sun et al. 2001), experience recombination (Jiggins et al. 2001; Werren and Bartos 2001; Jiggins 2002; Reuter and Keller 2003), undergo horizontal transfer to new insect hosts (Werren, Zhang, and Guo 1995; Heath et al. 1999; Vavre et al. 1999; Dyson, Kamath, and Hurst 2002; Kondo et al. 2002), and harbor many mobile elements (Masui et al. 1999; Masui et al. 2000; Wu et al. 2004). The genetic factors that underlie this level of genomic plasticity in endosymbionts remain unclear, although the A-*Wolbachia* genome is uniquely littered with insertion sequence elements, retrotransposons, and prophages (Wu et al. 2004). If mobile elements such as WO figure prominently in promoting recombination, they could increase genome size and transfer *Wolbachia* chromosomal DNA into recipient genomes. Perhaps just as bacteriophages of *E. coli* shuttle virulence genes and drive genomic divergence, bacteriophages of *Wolbachia*, in part, shape the genomic and phenotypic plasticity in this endosymbiont.

To assess the potential of bacteriophages in endosymbiont genome evolution, we address the following three objectives. First we determine if bacteriophage WO is common to a broad set of *Wolbachia* genomes by screening for bacteriophage loci in 27 lineages of *Wolbachia* representing the taxonomic range of the A and B supergroups. Second, we examine horizontal transfer of WO between distantly related parasitic *Wolbachia*. WO lateral transfer was first reported between A and B *Wolbachia* strains that coinfect a lepidopteran host (Masui et al. 2000), and multiple infections are common in the *Wolbachia*-arthropod endosymbiosis (Werren, Windsor, and Guo 1995; Jeyaprakash and Hoy 2000; Werren and Windsor 2000). These findings, along with the early suggestion that recombination may occur between A and B *Wolbachia* (Werren, Zhang, and Guo 1995) and the discovery of a bacteriophage in a γ-Proteobacteria endosymbiont (van der Wilk et al. 1999; Sandstrom et al. 2001), collectively motivate the development of the "intracellular arena" hypothesis, in which genetic exchange can occur in communities of bacterial endosymbionts that coinfect the same host cellular environment. We test the "intracellular arena" hypothesis using phylogenetic and restriction digest analyses of phage WO in two host insect systems that are coinfected by A and B *Wolbachia* (the hymenopteran wasp *Nasonia vitripennis* and the dipteran fruit fly *Drosophila simulans*). Finally, we use sequence data to investigate recombination within and between WO genes, and to estimate recombination rates of these genes. We then ask how recombination rates of bacteriophage genes compare with those estimated for *Wolbachia* chromosomal genes.

The recent publication of the *Wolbachia* genome sequence revealed two divergent families of prophage WO, labeled WO-A and WO-B (Wu et al. 2004). This study strictly focuses on the latter, as primers used in this study would not amplify genes from WO-A, because the gene is missing or the sequence is too divergent. Results indicate that phage WO-B is a major source of genomic flux in the *Wolbachia* endosymbiont: it (1) is prevalent, (2) experiences recombination at higher rates than host bacterial genes, and (3) laterally transfers between *Wolbachia* genomes within a diverse set of insect cellular environments (Lepidoptera, Hymenoptera, and Diptera).

Materials and Methods

Isolation, Amplification, and Sequencing of Genomic DNA

Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen) from single adult females of the arthropod species listed in table 1. *Drosophila simulans* (*w*Ha), *Drosophila simulans* (*w*No), *Drosophila simulans* (*w*Au Coffs Harbor S20), and *Drosophila simulans* (*w*Ri Agadir) were kindly provided by S. Charlat and H. Mercot (Institut Jacques Monod, CNRS-Universites Paris). *Drosophila simulans* (*w*Ha) and *Drosophila simulans* (*w*No) were obtained by a segregation experiment from a naturally double-infected stain from the Seychelles Archipelago (Poinsot, Montchamp-Moreau, and Mercot 2000). *Drosophila innubila* (In-2) and *Drosophila recens* (BM23) DNA were provided by J. Jaenike (University of Rochester). *Culex pipiens* and *Culex quinquefasciatus* were provided by J. Rasgon (University of California at Davis). DNA samples of *Asobara tabida*, *Encarsia formosa*, *Muscidiforax uniraptor*, *Protocalliphora sialia* (00-175), *Protocalliphora sialia* (00-189), and *Trichopria Drosophilae* were provided by J. Werren (University of Rochester), and DNA samples of *Drosophila melanogaster* (*w*Mel Popcorn), *Armadillidium vulgare*, *Bemisia tabaci*, *Drosophila teissieri*, *Porcelio werneri*, and *Tribolium castaneum* were provided by K. Bourtzis (University of Ioannina). *Nasonia vitripennis* A (strain 12.1) and B (strain 4.9) were derived from a segregation experiment from a *Nasonia vitripennis* strain naturally coinfected with both A and B *Wolbachia* (Perrot-Minnot, Guo, and Werren 1996); *Nasonia giraulti* A (strain 16.2) and *Nasonia longicornis* A (strain 2.1) were derived in a similar way

(unpublished). All DNA samples were diluted 1:10 and 1:100 in low TE (10 mM Tris, 0.5 mM EDTA) to serve as template for polymerase chain reactions (PCRs).

Bacteriophage WO-B and *Wolbachia* host chromosomal DNA were amplified using PCR in a volume of 10 μl (2.5 mM $MgCl_2$ [Promega], 0.25 mM of each dNTP [Invitrogen], 0.4 mM of the forward and reverse primer, 0.2 U Taq polymerase [Promega], and 1 ml DNA, 1 ml 10× PCR buffer [Promega], and 4.36 ml of water (ICN Biomedicals)). Primers for bacteriophage ORF7 and the *Wolbachia* surface protein (*wsp*) have been previously described (Zhou, Rousset, and O'Neill 1998) and are specific to WO-B and *Wolbachia*, respectively. Primers for bacteriophage ORF2 were designed by searching for homologous regions in the aligned ORF2 prophage WO-B sequences from the *Wolbachia* of *Ephestia kuehniella* (*w*Kue [Masui et al. 2000]) and *Drosophila melanogaster* (*w*Mel; sequence data were obtained from The Institute for Genomic Research Web site at http://www.tigr.org). ORF2WOF153 (5′-CATGGATTCACTTTCTCCGTCA-CC-3′) and ORF2WOR899 (5′-GGACTATAAAGACTT-GAAAGATG-3′) amplified a 747-bp fragment under the following reaction conditions for a limited set of DNA samples: 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min.

Sequencing was either performed directly from PCR products or from products cloned into plasmid vectors using the TOPO TA cloning kit (Invitrogen) and electrocompetent cells. All sequence templates were obtained from singly infected arthropod hosts (i.e., A or B *Wolbachia* infected). Amplified products of ORF7 were generally cloned because of heterogeneous copies within arthropod hosts. The number of clones sequenced per infection ranged from 12 to 40, with an average of 25. Confirmed PCR products and clones were sequenced bidirectionally using appropriate primers on an ABI 3700 or an ABI 3730 automated sequencer using Big Dye version 3.0 (Applied Biosystems).

All new sequences were deposited in GenBank under accession numbers AY622487 to AY622504 (ORF7), AY622505 to AY622510 (ORF2), and AY622511 to AY622512 (*wsp*).

Table 1
Distribution of Bacteriophage WO

Host species	*Wolbachia* Infection	Bacteriophage PCR Product (±)
Asobara tabida wAtab1	A	+[a]
Asobara tabida wAtab3	A	+[a]
Camponotus pennsylvanicus	A	+
Camponotus chromaoides	A	+
Drosophila innubila	A	−
Drosophila melanogaster CS	A	+
Drosophila melanogaster wMel	A	+[b]
Drosophila melanogaster wMel Popcorn	A	+
Drosophila recens	A	−
Drosophila simulans wHa	A	+
Drosophila simulans wAu	A	+
Drosophila simulans wRi	A	+[c]
Drosophila simulans wCof	A	+[c]
Drosophila teissieri	A	+
Ephestia cautella	A	+[c]
Ephestia kuehniella	A	+[c]
Leptopolina heterotoma wLhet1	A	+[a]
Leptopolina heterotoma wLhet2	A	+[a]
Leptopolina heterotoma wLhet3	A	+[a]
Muscidiforax uniraptor	A	+
Nasonia vitripennis	A	+
Nasonia giraulti	A	+
Nasonia longicornis	A	+
Protocalliphora sialia 00-175	A	+
Trichopria drosophilae	A	−
Armadillidium vulgare	B	+
Bemisia tabaci	B	+
Corcyra cepharonica	B	+[c]
Culex pipiens	B	+
Culex quinquefasciatus	B	+
Drosophila simulans wNo	B	+
Drosophila simulans wMa	B	+
Encarsia formosa	B	+
Ephestia cautella	B	+[c]
Nasonia vitripennis	B	+
Porcelio werneri	B	+
Protocalliphora sialia 00-189	B	−
Teleogryullus taiwanemaa	B	+[c]
Tribolium castaneum	B	+
Drosophila melanogaster	0	−
Nasonia vitripennis	0	−
Water control	0	−
No DNA control	0	−

[a] Bacteriophage presence previously reported in Gavotte et al. (2004).
[b] Bacteriophage presence previously reported in Wu et al. (2004).
[c] Bacteriophage presence previously reported in Masui et al. (2000).

Restriction Digestion

A polymorphism specific to the shared ORF7 sequences from the A and B *Wolbachia* of *Nasonia vitripennis* was genotyped by *BseRI* (New England Biolabs) restriction enzyme digestion. Individual reactions consisted of 4 μl of the ORF7 PCR product, 1 μl of Reaction Buffer 4, 1 μl of enzyme, and 4 μl of distilled water and were incubated for 100 min at 37°C. After digestion, products were run on a 1% agarose gel to determine the presence and size of digested DNA. A 1-kb and 100-bp DNA Ladder (Invitrogen) were coelectrophoresed as size standards.

Sequence Nomenclature, Alignments, and Analyses

Sequences are identified by the name of the host arthropod species, followed by a letter representing the supergroup of the *Wolbachia* strain (A or B) and a number designating each unique family of bacteriophage WO-B. Accession numbers from previously published sequences are shown adjacent to each taxon. Sequences were assembled in Sequencher version 4.1.2, checked manually, and any ambiguous base calls were changed to N and were treated as missing data. Translated amino acid sequences were aligned in ClustalX (Thompson et al. 1997) and manually edited in MacClade version 4.05 (Maddison and Maddison 2002). Sequences obtained from cloning were considered unique and used in the data analyses if they showed greater than 1.5% sequence dissimilarity from the common set of clones. This was a conservative approach to reduce the use of clones resulting from any DNA replication errors during PCR, sequencing, and cloning procedures. The DNAsp version 3.99 program was used for analyses of nucleotide divergence (Rozas and Rozas 1999).

We evaluated recombination using two methods. First, we tested for significant topological differences between gene trees using the Shimodaira-Hasegawa (SH) test, a nonparametric maximum-likelihood–based test (Shimodaira and Hasegawa 1999) that is appropriate for cases when the trees under comparison are estimated from the data (Goldman, Anderson, and Rodrigo 2000). The second method is a population genetic approach implemented by LDhat (McVean, Awadalla, and Fearnhead 2002), a program that analyzes correlations between linkage disequilibrium (LD) and the physical distance for pairs of segregating sites. We also used this approach to estimate the rate of recombination ($2N_er$) using an approximated likelihood method under a coalescent framework. All data sets were run through four models, including a crossing-over model with the respective gene length, and a gene conversion model with 100-bp, 500-bp, and 2,500-bp tracts of recombination. Because recombination tract lengths are unknown for *Wolbachia* and the estimates of $2N_er$ are highly dependent on the recombination tract lengths, recombination rates from the genetic exchange model producing the best-likelihood score are presented and should be interpreted with some caution. Nonetheless, all data sets produced the best-likelihood score with a gene conversion, 100-bp tract model, except for the two ORF7 data sets, which produced the best score under the crossing-over model. In addition, the selected tract length for recombination had little effect on the relative magnitude of $2N_er$ in the different genes. The analyses included sites strictly with two segregating alleles and did not exclude rare alleles or incorporate sites with gaps or ambiguous bases. The analyses were repeated with only those sites determined informative for recombination by a coalescent method, and it did not qualitatively affect the significance of detecting recombination.

Phylogenetic Analyses

Maximum-likelihood (ML) and Bayesian methods were used to infer phylogenetic relationships. Before ML analyses, a DNA substitution model for each data set was selected using Modeltest version 3.06 (Posada and Crandall 1998) and the Akaike information criterion (AIC). ML heuristic searches were performed using 100 random taxon addition replicates with tree bisection and reconnection (TBR) branch swapping. ML bootstrap support was determined using 100 bootstrap replicates, each using 10 random taxon addition replicates with TBR branch swapping. Searches were performed in parallel on a Beowulf cluster using the clusterpaup program (A.G. McArthur, jbpc.mbl.edu/mcarthur) and PAUP* version 4.0b10 (Swofford 2002). Data sets were also analyzed with Bayesian phylogenetic methods using noninformative prior probabilities. These searches utilized a GTR model with unequal base frequencies, portion of invariant sites estimated from the data, and varying rates for each of the codon positions. The Markov chain Monte Carlo (MCMC) chains were started from a random tree and run for 2 million generations (MrBayes version 3.0b4 [Ronquist and Huelsenbeck 2003]). Trees were sampled every 100 generations, and a consensus tree was built on all trees with the exclusion of the first 1,000 trees (burn-in). Posterior probabilities were determined by constructing a 50% majority-rule tree of all the trees sampled.

Statistical Tests of Phylogenetic Congruence

We tested the significance of topological differences in phylogenetic trees using the SH test (Shimodaira and Hasegawa 1999). The SH test compares the likelihood score (–lnL) of a given data set across its ML tree versus the −lnL of that data set across alternative topologies, which in this case are the ML phylogenies for other data sets. The differences in the −lnL values are evaluated for statistical significance using bootstrap (1,000 replicates) based on RELL sampling and the more extensive full optimization (PAUP* version 4.0b10). These two approaches yielded similar results.

Results

Prevalence of a Bacteriophage in *Wolbachia*

Bacteriophage WO-B is known to be present in 13 lineages of the bacterial endosymbiont *Wolbachia* (Masui et al. 2000; Gavotte et al. 2004; Wu et al. 2004). We have PCR-screened an additional 26 lineages, composed of 15 A and 11 B *Wolbachia*. Results for bacteriophage presence are shown in table 1. Of the 26 lineages, 22 amplified an ORF7 PCR product of the expected fragment size. The four DNA samples that failed to amplify a product were screened with several DNA template concentrations (1×, 1:10×, 1:100×) and general 16S rDNA *Wolbachia* primers as a control for presence of the bacterial host chromosome. Only the positive control reactions for these DNA templates successfully amplified a *Wolbachia* PCR product. Several negative control reactions for the ORF7 product, which included genomic DNA from two insects cured of their endosymbiont, distilled water in place of template, and no template, did not amplify a fragment. In total, bacteriophage WO-B infects 35/39 (89.7%) surveyed lineages, representing 22/25 (88.0%) A and 13/14 (92.9%) B *Wolbachia*.

Phylogeny of Bacteriophage ORF7

Phylogenetic reconstruction of a 380-bp region of the putative capsid protein ORF7 was performed using both ML and Bayesian methods. Tree topologies of the two approaches are generally congruent, and figure 1 shows the ML best tree with both ML bootstrap values and Bayesian posterior probabilities. Based on the ORF7 region, the phylogeny distinguishes three strongly supported groups of bacteriophage WO-B, labeled groups I, II, and III. Average ORF7 nucleotide divergence levels between the three groups were estimated using the Jukes and Cantor method and are high in comparison with other *Wolbachia* genes, (I-II: 20.6%, II-III: 18.4%, I-III: 30.0%), whereas average nucleotide divergences within groups I, II, and III are 6.8%, 8.0%, and 4.4%, respectively. A sliding window analysis of nucleotide divergences between the three groups indicates regional variation in nucleotide divergences—group I is most divergent from groups II and III in the 3′

region, and groups II and III are most divergent in the 5′ region (data not shown).

Lateral Phage Transfer

Two lines of evidence from the phylogenetic analyses support extensive lateral bacteriophage transfer between *Wolbachia* endosymbionts. First, the bacteriophage tree topology in figure 1 is not congruent with the known topologies of *Wolbachia* gene trees, indicating a lack of codivergence between the two genomes. To statistically test the topological incongruence between bacteriophage and endosymbiont phylogenies (figs. 1 and 2), we compared the ML phylogenies of two bacteriophage putative genes, ORF2 and ORF7, against the ML phylogeny of a *Wolbachia* host chromosomal gene, *wsp*, using the SH test. We note that although *wsp* is widely used as the only sensitive phylogenetic marker of *Wolbachia* diversity, it can recombine (Jiggins et al. 2001; Werren and Bartos 2001) and therefore may not produce the most reliable *Wolbachia* gene tree. The two phage ORFs are closely linked, separated by 4.2 and 2.7 kb in the prophage genomes of *Wolbachia* from *Teleogryllus taiwanemna* and *Ephestia kuehniella*, respectively (Masui et al. 2000). If bacteriophage transfer is extensive and reflected in the overall phylogenies, then we expect to reject topological congruence between the bacteriophage and bacteria data sets.

We restricted the analysis to seven strains of A *Wolbachia* that are infected with a single lineage of bacteriophage WO-B. Unambiguously, the ORF2 and ORF7 data sets showed highly significant differences against the *Wolbachia wsp* tree, and vice versa, indicating strong topological incongruence between the phylogenies of bacteriophage genes and the endosymbiont host chromosome (fig. 2 and table 2). In contrast, the ORF2 and ORF7 data sets were not significantly different, with the exception of one comparison—the ORF2 data set against the ORF7 tree topology (table 2). The cause of this slight difference is isolated to the *E. kuehniella* strain, which branches in different positions and with different levels of support in the two-phage phylogenies (fig. 2). Repeating the SH test after removing the *E. kuehniella* sequences from the data sets eliminated the significant difference between ORF2 and ORF7. These results, along with a viewing of the *Ephestia kuehniella* ORF7 sequence (AB036660), reveal that this region is a chimeric sequence, possibly generated from a recombination event (see below).

The second line of evidence of lateral phage transfer is that strains of the distantly related A and B *Wolbachia* (estimated time of divergence is approximately 60 MYA [Werren, Zhang, and Guo 1995]), which naturally co-occur in a single arthropod host, share identical bacteriophage ORF7 sequences (fig. 1). We cloned and sequenced ORF7 PCR products from A and B *Wolbachia* that coinfect the hymenopteran *Nasonia vitripennis* (Perrot-Minnot, Guo, and Werren 1996) and the dipteran *Drosophila simulans* (Poinsot, Montchamp-Moreau, and Mercot 2000). In both systems, as well as in the lepidopteran *Ephestia cautella* (Masui et al. 2000), we found that an ORF7 sequence was identical for the coinfecting A and B *Wolbachia* (fig. 1). The shared

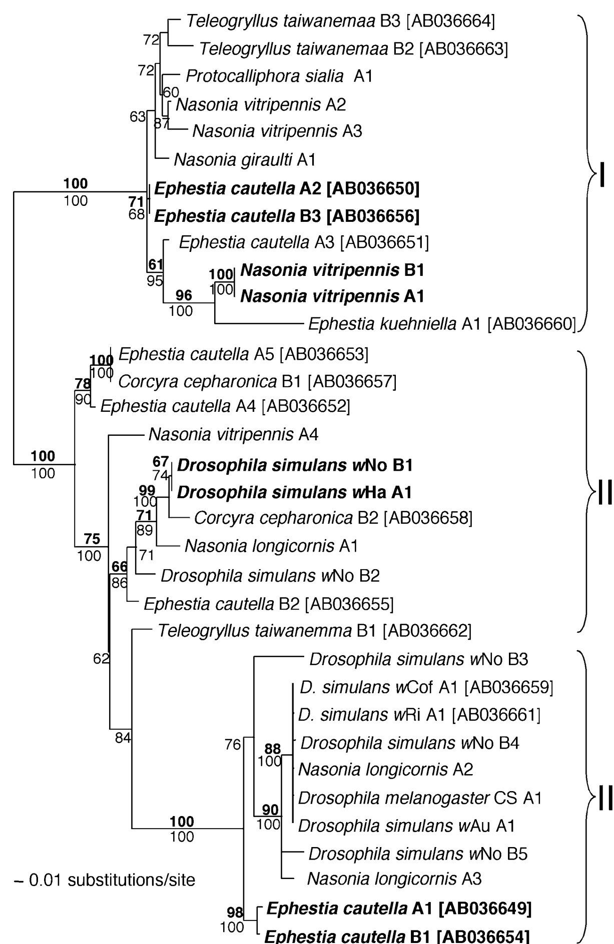

FIG. 1.—Maximum-likelihood (ML) phylogenetic tree of bacteriophage WO-B based upon sequences of the ORF7 region (putative capsid protein). The tree is midpoint rooted. Both ML bootstrap values (bold) and Bayesian posterior probabilities (plain) are shown. Name of the host arthropod species is followed by a letter denoting the *Wolbachia* supergroup (A or B) and a number designating each unique phage family. Pairs of adjacent taxa in bold represent inferred events of lateral phage transfer between A and B *Wolbachia* that coinfect the same arthropod host.

sequences were not derived from rare clones during the cloning process. In *N. vitripennis*, 5/22 clones for the A strain and 23/23 clones for the B strain yielded the shared sequence. In *D. simulans*, 27/27 clones for the A strain and 2/12 clones for the B strain yielded the shared sequence. We also cloned ORF7 PCR products from the singly infected *Nasonia giraulti* and *Nasonia longicornis* sibling species, in addition to *Protocalliphora sialia*, the dipteran host parasitized by *Nasonia*. As shown in figure 1, ORF7 sequences are not identical between these ecological associates, but some of them do cluster together in group I of the phylogeny.

ORF7 Restriction Digestion

A restriction digest analysis was performed to confirm the finding of lateral phage transfer between the A and B *Wolbachia* of *Nasonia*. The enzyme *BseRI* specifically cuts the shared bacteriophage ORF7 sequence of the *N. vitripennis* A and B *Wolbachia* into two fragments (fig. 3). Independent ORF7 PCR products from

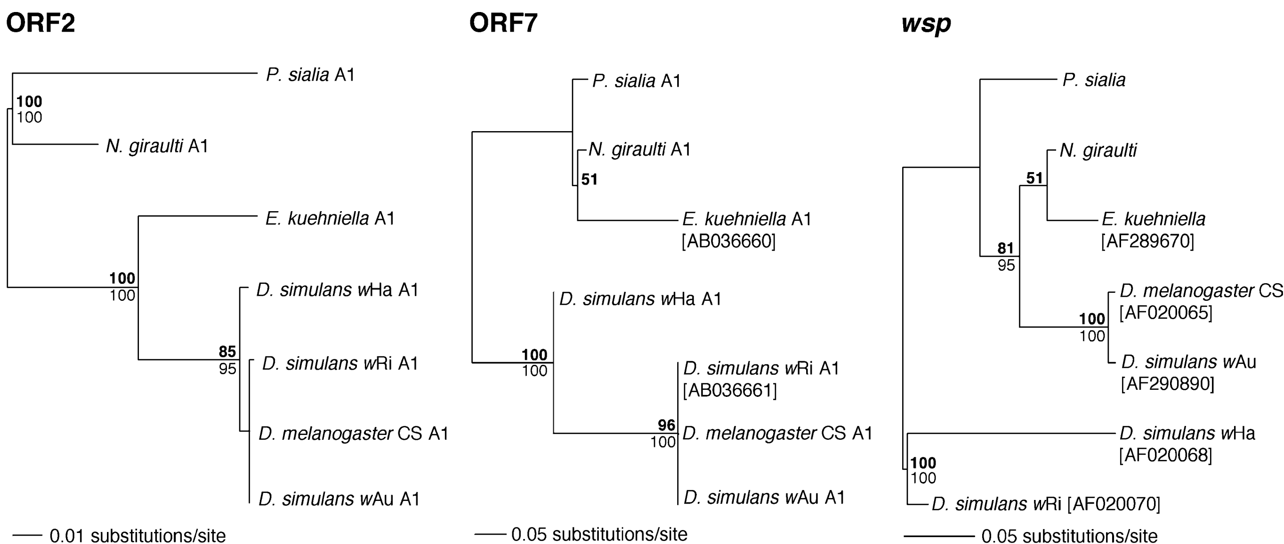

FIG. 2.—Comparison of bacteriophage and host endosymbiont ML trees. Phylogenetic trees of bacteriophage WO-B are based upon sequences of the ORF2 (putative DNA packaging protein) and ORF7 (putative capsid protein) regions; the phylogenetic tree of the host *Wolbachia* chromosome is based upon sequences of the *wsp* region. Each of the trees are midpoint rooted and estimated using maximum likelihood (ML). Bold numbers indicate ML bootstrap values and plain numbers indicate Bayesian posterior probabilities.

the same two DNA samples used for cloning and two new DNA samples freshly extracted from single adults of the same strains, were used as template for the restriction digestion. Negative controls consisted of a no-DNA water template and the ORF7 PCR product of *Drosophila melanogaster* that lacks the restriction site for *BseRI*. ORF7 PCR products for the *N. vitripennis* A strain also served as an internal negative control because only one of the multiple ORF7 sequences present in the PCR product (i.e., the shared sequence) contains the *BseRI* restriction site. Results are shown in figure 3 and confirm that the shared ORF7 sequence is cut into two smaller fragments of the expected size (259 bp and 141 bp) for both *N. vitripennis* A and B. The ORF7 PCR products of *D. melanogaster* (418 bp) and *N. vitripennis* A (400 bp) that lack the restriction site yield bands of the expected full fragment size.

Recombination Rates of Bacteriophage WO and *Wolbachia*

ORF7 and ORF2 are less than 5 kb apart in the prophage genomes of *Teleogryllus taiwanemna* and *Ephestia kuehniella*, respectively (Masui et al. 2000), yet results from the SH test above suggested a weakly significant topological incongruence between these two regions (fig. 2 and table 2). In particular, the phylogenetic positioning of *Ephestia kuehniella* was different in the two data sets, and a viewing of the *Ephestia kuehniella* ORF7

Table 2
Results of Shimodaira-Hasegawa Test of Alternative Tree Topologies for Bacteriophage Genes and a *Wolbachia* Chromosomal Gene

	Data Set		
Topology	Phage ORF2	Phage ORF7	*Wolbachia wsp*
Phage ORF2	**1595.52**	1142.02	1825.35****
Phage ORF7	1611.02*	**1141.44**	1822.02****
Wolbachia wsp	1623.84****	1264.50****	**1744.59**

NOTE.—The −Ln likelihood scores of phylogenetic reconstructions are shown for bacteriophage and *Wolbachia* gene regions. The lowest (best) likelihood scores for each data set are indicated in boldface. Significance levels are based on full optimization and indicated as follows: * $P < 0.05$, **** $P < 0.0001$.

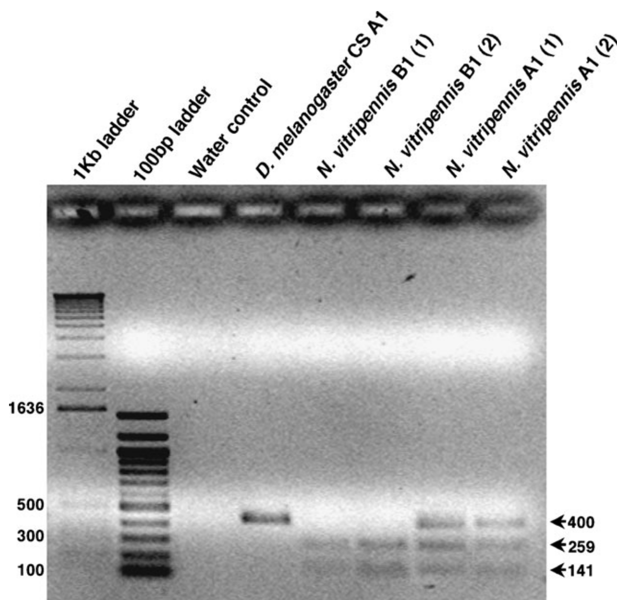

FIG. 3.—Confirmation of shared bacteriophage sequence between A and B *Wolbachia* in the parasitic wasp *Nasonia vitripennis*. ORF7 PCR products were digested using the *BseR1* enzyme. Name of the host arthropod species is followed by a letter representing the *Wolbachia* supergroup (A or B) and a number designating the family of bacteriophage WO-B. For *N. vitripennis*, the number 1 indicates that the DNA template came from the same source as that used in generating the sequences for figure 1, and the number 2 indicates that the DNA template came from independently extracted DNA from the same insect strain. Results are identical for both templates.

Nucleotide position	2	6	8	13	14	15	18	22	23	29	30	32	35	36	38	43	48	50	53	54	65	68	72
Group I	A	C	G	G	C	T	C	A	A	A	G	C	A	C	A	A	T	C	C	C	A	T	C
E. kuehniella A1	A	T	A	A	C	C	A	G	C	G	A	T	T	T	**G**	G	C	G	T	G	A	T	C
Group III	G	T	A	A	T	C	A	G	C	G	A	T	T	T	A	G	C	G	T	G	G	G	A

Nucleotide position	74	76	77	80	84	87	89	95	99	100	101	104	113	125	135	140	143	149	159	161	166	167	184
Group I	C	A	T	T	A	C	A	C	T	C	A	G	G	C	G	A	A	T	G	T	C	A	T
E. kuehniella A1	A	G	C	T	A	G	A	C	T	C	A	G	**T**	**G**	G	A	A	**A**	G	T	C	A	T
Group III	A	G	C	C	G	G	G	A	A	A	T	T	G	C	A	G	G	T	A	G	T	T	C

Nucleotide position	185	188	191	194	195	197	205	206	208	210	212	214	216	217	218	224	227	228	233	234	252	253	254
Group I	T	C	C	T	C	T	G	G	G	G	T	G	A	T	G	A	T	G	G	T	A	T	T
E. kuehniella A1	T	C	T	T	C	T	G	G	G	G	T	G	A	T	G	A	T	A	G	T	A	**C**	T
Group III	C	T	T	C	A	C	T	T	C	A	G	A	G	A	A	T	A	A	C	C	G	A	A

Nucleotide position	255	256	257	260	272	274	278	280	281	285	288	290	293	294	300	312	317	329	331	332	347	354	356
Group I	G	A	G	G	C	A	T	G	A	A	T	A	G	A	A	G	A	G	A	G	A	G	A
E. kuehniella A1	G	A	G	**A**	C	**G**	T	G	A	A	**G**	A	G	A	A	G	A	G	A	**A**	A	G	C
Group III	A	C	T	T	G	A	A	C	T	G	C	T	A	T	C	A	G	A	G	G	T	A	C

Nucleotide position	363	369	374	375	380
Group I	A	A	A	C	A
E. kuehniella A1	A	C	**C**	A	G
Group III	G	C	A	A	G

FIG. 4.—Sequence variation in ORF7 showing that the *E. kuehniella* A1 region is a putative chimeric sequence between group I (represented by *Nasonia vitripennis* A1) and group III (represented by *Drosophila simulans* wNo B3). Group I and group III are shaded white and gray, respectively, and the sequence homologies shared with *E. kuehniella* A1 are colored accordingly. Substitutions not shared with either group are noted in bold and indels are deleted.

sequence (AB036660) suggests a recombination event in this lineage; it is a chimeric sequence that shares homology with the first 64 bp of group III sequences and with the last 316 bp of group I sequences (fig. 4). This observation led us to further investigate recombination.

To statistically detect recombination among ORF2, ORF7, and *wsp* under a population genetic framework (Werren and Bartos 2001; Reuter and Keller 2003), we employed the LDhat program (McVean, Awadalla, and Fearnhead 2002). As shown in table 3, this program estimates (1) the population mutation rate (θ_W), (2) the correlation coefficients of linkage disequilibrium (LD) with distance, (3) the significance of the correlation with three different permutation tests, and (4) the population recombination rate ($2N_er$) per locus under a coalescent framework. Recombination predicts a significantly negative relationship between LD of segregating sites and distance, and of the two measures of LD, r^2 tends to be a more sensitive method. As shown for the ORF2, ORF7, and *wsp* regions with the smaller data sets ($N = 7$), which correspond to those used in figure 2 and table 2, significant recombination is unambiguously present in all three regions. We also repeated the analysis with the complete ORF7 data set and a broader *wsp* data set previously used (Jiggins 2002). Significant recombination is again evident in the larger bacteriophage ORF7 data set, the *wsp* A and B data sets, and the *wsp* homolog data set in the related *Ehrlichia ruminantium*, but not in the *wsp* data set of the nematode *Wolbachia*.

The population rates of recombination ($2N_er$) per locus are presented in table 3 and indicate strong differences for bacteriophage and endosymbiont genes. Recombination rates vary from 0.2 in the nonrecombining nematode *Wolbachia* to 15.0 in the bacteriophage ORF7. The A and B *Wolbachia* chromosomal gene *wsp* and the bacteriophage region ORF2 show low to moderate recombination rates (0.6 to 4.0). To compare recombination rates between genes, it is helpful to control for variation in population sizes (N_e) that can affect the estimates of recombination rate. The ratio $2N_er/\theta_W$ (per site) reduces to $2N_er/2N_eu$ and then to r/u, yielding the likelihood of a base pair experiencing a recombination event relative to mutation in a given gene. These ratios vary from 0.004 in the nematode *Wolbachia* to 0.439 in

Table 3
Recombination in Bacteriophage WO and Wolbachia

					Correlation		Permutation Tests				
Taxa	Gene	Length	Number	θ_W	r^2, d	$\lvert D'\rvert$, d	P_{lk}	Pr^2	$P_{\lvert D'\rvert}$	$2N_er$/Locus ($-$lnL)	$2N_er$/Site/θ_W
Bacteriophage WO	ORF7	365	7	0.132	−0.506	−0.306	0.000	0.000	0.000	10.2 (−61109.7)	0.211
Bacteriophage WO	ORF2	703	7	0.064	−0.076	−0.062	0.000	0.001	0.007	0.6 (−62326.9)	0.014
Wolbachia (A group)	wsp	602	7	0.092	−0.128	−0.064	0.000	0.000	0.000	1.0 (−83094.1)	0.018
Bacteriophage WO	ORF7	380	34	0.090	−0.274	−0.212	0.000	0.000	0.000	15.0 (−228648.5)	0.439
Wolbachia (A group)[a]	*wsp*	570	17	0.069	−0.110	0.021	0.000	0.000	0.778	3.8 (−141605.0)	0.097
Wolbachia (B group)[a]	*wsp*	570	35	0.086	−0.049	−0.028	0.000	0.000	0.114	4.0 (−429754.3)	0.082
Wolbachia (Nematodes)[a]	*wsp*	444	10	0.122	−0.052	−0.016	0.101	0.052	0.297	0.2 (−121946.4)	0.004
Ehrlichia ruminantium[a]	Map1	870	14	0.069	−0.012	−0.034	0.000	0.167	0.060	3.2 (−279157.1)	0.053

[a] Data set is from Jiggins (2002).

bacteriophage ORF7 and indicate that sites in the bacteriophage ORF7 region have a greater likelihood of experiencing recombination than do sites in any other gene. The *r*/*u* value for ORF7 is at least fourfold greater than that of *wsp* in the A and B *Wolbachia*, 118-fold greater than that of *wsp* in the nematode *Wolbachia*, and eightfold higher than that of a *wsp* homolog in the related bacterium, *E. ruminantium*.

We note here that our estimates of recombination rate per locus in the larger *wsp* data set are in most cases dramatically lower than those previously estimated using the same data set (Jiggins 2002). We have discussed these differences with the author and have determined that the rates of recombination were overestimated as a linear chromosome model was assumed, instead of the more appropriate circular genome model for bacteria. The corrected estimates of $2N_er$ are presented here, whereas the other analyses and conclusions remain unaffected.

Discussion

The specialized genomes of intracellular bacterial endosymbionts often lack one of the major contributors of genomic flux in the free-living microbial world—bacteriophages. However, this study yields several results that show bacteriophages have influenced the genome evolution of one of the most widespread bacterial endosymbionts in the biosphere, *Wolbachia*. First, bacteriophage WO is abundant and widespread, occurring in at least 89% of the sampled endosymbiont lineages. Second, bacteriophage WO laterally transfers between very divergent lineages (i.e., the A and B *Wolbachia*) and uses multiply infected host cells as an intracellular arena for horizontal movement. Third, bacteriophage regions undergo recombination, and, in the case of the putative capsid protein ORF7, the recombination rate is the fastest reported rate for this endosymbiont genome.

Our results build upon findings from other papers (Masui et al. 2000; Masui et al. 2001; Wu et al. 2004) and further indicate that bacteriophages can be significant contributors to genomic flux in a major group of bacterial endosymbionts. Besides a single plasmid of the aphid mutualist *Buchnera* that was putatively acquired by lateral transfer (Van Ham et al. 2000), bacteriophage WO is the only other element shown to laterally move in obligate bacterial endosymbionts. Even among the broader groups of nonobligate, or facultative, intracellular bacteria, such as the mammalian pathogens *Bordetella*, *Chlamydia*, and *Mycoplasma*, prophages are known to be present in their genomes (Tu et al. 2001; Karunakaran et al. 2002; Parkhill et al. 2003), yet lateral transfer remains to be shown.

As much as the presence of horizontal gene transfer (HGT) is a hallmark of genome evolution in free-living bacteria, its absence has become a hallmark of genome evolution in primary endosymbionts, obligate mutualists that form stable, long-term host associations. The highly reduced genomes and sheltered lifestyles of these obligate intracellular bacteria present negligible opportunities for gene acquisition events and even further confinement to genome degradation. For example, the well-studied *Buchnera* endosymbiont has experienced almost no HGT with the exception of a putative plasmid acquisition (Van Ham 2000). *Buchnera aphidicola* genomes estimated to have diverged 50 to 70 MYA have identical genomic architectures and no indication of inversions, translocations, duplications, and HGTs (Tamas et al. 2002). Additionally, many endosymbionts have experienced extensive loss of genes that encode DNA repair and recombinase functions (Moran and Wernegreen 2000), which further reduce the opportunities for genomic flux.

Are the genomes of bacterial endosymbionts sealed to a fate of extraordinary genome stability? The answer apparently depends on the specific lifestyle of the endosymbiont. Findings of phage transfer and recombination suggest a clear mechanism for foreign DNA acquisition in *Wolbachia*. Whereas primary endosymbionts such as *Buchnera* are strictly obligate mutualists, vertically transmitted, confined to a limited host range, and subject to the restraints of specialized host cells termed bacteriocytes, secondary endosymbionts and reproductive parasites such as the arthropod A and B *Wolbachia* can be parasitic, horizontally transmitted, able to infect a broader host range, and not typically enclosed in bacteriocytes. Because HGT rates are expected to depend on the availability of foreign DNA and the probability of successful foreign DNA introduction without being lost by genetic drift (Lawrence 1999), we predict that the more labile nature of secondary symbionts and reproductive parasites predisposes their genomes to higher rates of HGT and overall genome instability. With the lingering possibility that the APSE-1 phage of the secondary symbiont(s) of aphids (van der Wilk et al. 1999; Sandstrom et al. 2001) may show similar levels of flux, it will likely become increasingly apparent to forge different forecasts on how the genomes of primary and other endosymbionts evolve.

The *Wolbachia* genome sequence from *Drosophila melanogaster* is a striking example in this regard (Wu et al. 2004). Although the genome sequence shows evidence of severe reductive evolution consistent with the small genomes of other obligately, intracellular bacteria, the amount of genome flux, including rearrangements, duplications, and repetitive and mobile element DNA (greater than 14% of the genome) is unequaled in other endosymbiont genomes. In fact, the genome architecture of *Wolbachia* looks more like that of a free-living bacteria than other intracellular bacteria.

One important question for the future is how widespread these mobile elements are in other *Wolbachia* strains. The work here suggests that at least some of these mobile element regions are very widespread throughout the parasitic *Wolbachia*. Another important question is why the *Wolbachia* genome has so much more repetitive and mobile DNA. Wu et al. (2004) explain that similar to other intracellular species, the efficacy of selection might be reduced in *Wolbachia* because of genetic drift and population bottlenecks, thereby permitting the maintenance of these mobile elements throughout the radiation of the supergroups. However, such elements are completely missing from some of the most reduced endosymbiont genomes (i.e., *Buchnera*, *Wigglesworthia*, and *Blochmannia*) and are thought to be readily lost from genomes experiencing reductive evolution.

Although the persistence of large amounts of mobile DNA in *Wolbachia* (~14%) may be assisted by relaxed selection, the invasion of so much mobile DNA is probably caused by recurrent exposure to mobile element gene pools such as phages (Wu et al. 2004). Our findings indicate that mobile element exchange can be a frequent and ongoing process in the *Wolbachia* genome. A bacterial system that readily transfers across hosts and coinfects with other strains and groups of bacteria leads to a greater (1) availability of foreign DNA and (2) probability of successful foreign DNA introduction. Such community dynamics (within the intracellular arena) increase the rates of HGT into *Wolbachia*, promote the spread of new genetic parasites, and are in stark contrast to the confined community dynamics of primary insect endosymbionts, which notably lack mobile elements.

Although the main mode of endosymbiont transmission is vertical from mother to offspring, endosymbionts such as *Wolbachia* readily transfer to new arthropod hosts (Werren, Zhang, and Guo 1995; Heath et al. 1999; Vavre et al. 1999) and consequently create new opportunities for foreign DNA introduction. *Wolbachia*-infected arthropod hosts commonly harbor two major supergroups of *Wolbachia* (up to 34.6% AB coinfection [Werren and Windsor 2000]) that have an estimated divergence time of approximately 60 MYA (Werren, Zhang, and Guo 1995). Multiple infections also occur with different strains of the same supergroup, with as many as five different *Wolbachia* occurring in the ant *Formica exsecta* (Reuter and Keller 2003). More interestingly, *Wolbachia* coinfect arthropods with independently derived bacterial associates of arthropods such as the recently discovered *Cytophaga*-like organism (a.k.a., CLO) (3.1% coinfection, [Weeks, Velten, and Stouthamer 2003]) and various primary endosymbionts of insects such as *Blochmannia* of the ant genus *Camponotus* (Bordenstein and Wernegreen, unpublished data). HGT between endosymbionts coinfecting the same intracellular arena could be a dramatic source of evolutionary novelty for bacterial genomes confined to an intracellular lifestyle.

Do bacteriophages move between distantly related endosymbiont genomes? It is premature to say what kinds of genomic distances bacteriophages cross in endosymbiotic bacteria. Our data clearly support the intracellular arena hypothesis in which bacteriophages can jump between ancient strains of the same bacterial genus in the same cytoplasmic environment. Of particular interest is that bacteriophage WO is actually the first genetic element known to traverse the divergent boundaries of the A and B *Wolbachia* supergroups. Backed by consistent genetic evidence, the two groups have been considered discrete lineages and impermeable to each other (i.e., no recombination or gene exchange). However, the phylogenetic data confirm that WO has readily transferred between the supergroups (fig. 1). This transfer likely occurs at appreciable rates given the phylogenetic discordance between WO and *Wolbachia* trees (table 2) and the abundance of WO throughout *Wolbachia* (table 1). The combination of this molecular data also implicitly suggests that WO is indeed an active bacteriophage or has been so in the recent evolutionary past. Data on the presence of bacteriophage WO gene expression (Masui et al. 2000) and particle enrichment (Masui et al. 2001; Fujii et al. 2004) also support the activity of this bacteriophage.

If WO is active, lytic, and moves between *Wolbachia* that have traditionally been considered discrete genetic lineages, then bacteriophages might also transfer between unrelated lineages of endosymbionts in the same cellular environment. It is noteworthy that although CLO and *Wolbachia* are independently derived from different groups of eubacteria (i.e., the Cytophaga-Flavobacteria-Bacteriodes and α-Proteobacteria, respectively), they share the ability to induce reproductive alterations such as parthenogenesis (Zchori-Fein et al. 2001), feminization (Weeks, Marec, and Breeuwer 2001), and cytoplasmic incompatibility (Hunter, Perlman, and Kelly 2003). Although the genetic machinery underlying these reproductive alterations remains elusive, the intracellular arena hypothesis (perhaps via bacteriophage exchange) could provide a parsimonious explanation for the evolution of reproductive parasitism in vastly different groups of eubacteria that coincidentally infect the same arthropod hosts.

In summary, the findings here show that in one of the most abundant bacterial endosymbionts in the biosphere (*Wolbachia*), a bacteriophage is prevalent, recombines, and undergoes recent transfers in coinfected hosts. Bacteriophages, therefore, can penetrate the intracellular lifestyle of bacterial endosymbionts and play an active role in their genome evolution, perhaps even similar to the roles they play in free-living bacterial systems.

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