Complete Bacteriophage Transfer in a Bacterial Endosymbiont (Wolbachia) Determined by Targeted Genome Capture

Bethany N. Kent¹, Leonidas Salichos¹, John G. Gibbons¹, Antonis Rokas¹, Irene L. G. Newton², Michael E. Clark³, and Seth R. Bordenstein*,¹

¹Department of Biological Sciences, Vanderbilt University
²Department of Biological Sciences, Wellesley College
³Department of Biology, University of Rochester

*Corresponding author: E-mail: s.bordenstein@vanderbilt.edu.

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Abstract

Bacteriophage flux can cause the majority of genetic diversity in free-living bacteria. This tenet of bacterial genome evolution generally does not extend to obligate intracellular bacteria owing to their reduced contact with other microbes and a predominance of gene deletion over gene transfer. However, recent studies suggest intracellular coinfections in the same host can facilitate exchange of mobile elements between obligate intracellular bacteria—a means by which these bacteria can partially mitigate the reductive forces of the intracellular lifestyle. To test whether bacteriophages transfer as single genes or larger regions between coinfections, we sequenced the genome of the obligate intracellular Wolbachia strain wVitB from the parasitic wasp Nasonia vitripennis and compared it against the prophage sequences of the divergent wVitA coinfection.

We applied, for the first time, a targeted sequence capture array to specifically trap the symbiont's DNA from a heterogeneous mixture of eukaryotic, bacterial, and viral DNA. The tiled array successfully captured the genome with 98.3% efficiency. Examination of the genome sequence revealed the largest transfer of bacteriophage and flanking genes (52.2 kb) to date between two obligate intracellular coinfections. The mobile element transfer occurred in the recent evolutionary past based on the 99.9% average nucleotide identity of the phage sequences between the two strains. In addition to discovering an evolutionary recent and large-scale horizontal phage transfer between coinfecting obligate intracellular bacteria, we demonstrate that “targeted genome capture” can enrich target DNA to alleviate the problem of isolating symbiotic microbes that are difficult to culture or purify from the conglomerate of organisms inside eukaryotes.

Key words: horizontal gene transfer, endosymbiont, intracellular.

Introduction

Horizontal gene transfer (HGT) permits the acquisition of novel DNA sequences and adaptations through “the non-genealogical transmission of genetic material from one organism to another” (Goldenfeld and Woese 2007). In free-living bacteria, the rate of occurrence of HGT is very high and nearly universal. In contrast, obligate intracellular bacteria have few opportunities to acquire new genes due to being reproductively confined to a eukaryotic host cell. As a result of their restrained lifestyle, genome evolution of obligate intracellular bacteria is dominated by gene loss rather than gene insertion (Ochman et al. 2000; Mira et al. 2001, 2002; Andersson et al. 2002; Bordenstein and Reznikoff 2005). For instance, the pervasive amount of HGT in free-living bacteria contrasts with the extreme stability and downsizing observed for the small endosymbiont genomes of aphids (Shigenobu et al. 2000; Tamas et al. 2002; van Ham et al. 2003; Degnan et al. 2005), ants (Gil et al. 2003; Degnan et al. 2005), and flies (Akman et al. 2002), in which no rearrangements or inflow of genetic material have occurred over millions of years.

HGT can be driven by a genetic vehicle that transfers genes from one cell to another, typically in the form of bacteriophages, plasmids, or transposons (Frost et al. 2005). In contrast to the highly reduced genomes of host-restricted
species that tend to manifest striking degrees of stability and gene synteny, obligate intracellular bacteria that host-switch often harbor mobile elements and the genetic toolkit to acquire new DNA (Bordenstein and Reznikoff 2005; Newton and Bordenstein 2010). Examples of extrachromosomal mobile elements in ancient, horizontally transmitted obligate intracellular bacteria include Rickettsial plasmids (Baldridge et al. 2010) and an integrative conjugative element (ICE; Blanc et al. 2007), Phytoplasma plasmids (Oshima et al. 2001) and Chlamydiaceae phages (Hsia et al. 2000; Read et al. 2000).

In free-living bacteria, bacteriophages are significant to the structure of bacterial ecosystems and adaptive evolution. Phage-mediated HGT (Ochman et al. 2000; Bushman 2002; Canchaya et al. 2003) accounts for the majority of intraspecific genome diversification among environmental bacteria and human pathogens (Ohnishi et al. 2001; Banks et al. 2002; Van Sluys et al. 2003). In contrast, the transfer of a full bacteriophage genome between the chromosomes of obligate intracellular bacteria in the same host has not been demonstrated. Of the obligate intracellular bacteria, Wolbachia is a likely target for large-scale HGT of mobile elements because up to 21% of the genome can be dedicated to mobile DNA, specifically transposons and the Wolbachia bacteriophage family, known as WO (Wu et al. 2004; Klasson et al. 2008; Klasson et al. 2009; Kent and Bordenstein 2010). Wolbachia are a genus of cytoplasmically transmitted r-proteobacteria and are among the most common bacterial infections in arthropods (Werren and Windsor 2000; Hilgenboecker et al. 2008). Recent interest in these bacteria has emerged because of their ability to 1) induce the major inflammation responses associated with filarial diseases (Bandi et al. 2001; Nutman 2001; Taylor et al. 2001), 2) reduce vector capacity of mosquitoes and decrease populations of vectorial insects (Zabalou et al. 2004; Evans et al. 2009; McMeniman et al. 2009; Turley et al. 2009), 3) transfer genes to the genomes of their host animals (Kondo et al. 2002; Hotopp et al. 2007; Nikoh et al. 2008), 4) modify host sex ratios and sex determination mechanisms for their own advantage (Werren et al. 2008), and 5) influence arthropod speciation (Bordenstein et al. 2001; Bordenstein 2003; Jaenike et al. 2006; Koukou et al. 2006; Miller et al. 2010).

Although mobile elements have been identified in Wolbachia and other obligate intracellular bacteria, there is sparse evidence of recent, large-scale HGTs outside of insertion sequences (Cordaux et al. 2008). In Wolbachia, minor capsid gene sequences from bacteriophage WO are identical between coinfections in five different insect species (Masui et al. 2001; Bordenstein and Werren 2004; Chafee et al. 2010), suggesting that transfer between Wolbachia coinfections in the same host is very common. However, evidence of phage transfer beyond the ~350 bp of this single gene has not previously been shown and the question remains if the whole phage or just single genes can transfer between Wolbachia in the same host. The implication of large mobile element transfers between obligate intracellular coinfections is significant to bacterial genome evolution. For some intracellular infections, especially those that are not confined to a single host or restricted to bacteriocytes (Bordenstein and Reznikoff 2005; Kent and Bordenstein 2010), the exposure and ability to take up novel DNA sequences creates an opportunity for these bacteria to partially mitigate the reductive nature of an intracellular lifestyle.

To determine if a complete bacteriophage WO transfer occurred between coinfections, we sequenced the genome of the B-Wolbachia infection of the parasitic wasp Nasonia vitripennis (wVitB) and compared it with the prophage sequences of the A-Wolbachia (wVitA) that coinfects the same host. These two ancient infections diverged ~60 million years ago (Werren et al. 1995). In this study, the wVitA and wVitB infections were previously segregated into separate N. vitripennis strains to avoid DNA contamination. The wVitB and wVitA coinfections are likely candidates for phage transfer because 1) wVitB and wVitA share an identical minor capsid gene sequence (Bordenstein and Werren 2004); 2) active phage has been visualized inside wVitA bacterial cells by EM (Bordenstein et al. 2006); 3) these phage-containing Wolbachia showed structural defects including a collapsed inner membrane, degraded DNA, and lysis coupled with phage release (Bordenstein et al. 2006); and 4) the WOVitA1 phage is the only phage in the wVitA genome that encodes a full set of structural genes, including head, tail, and baseplate genes (fig. 1), indicating it is able to form active phage particles. If transfer of the complete phage occurs between wVitA and wVitB, then it is likely that the several other examples of phage gene transfer between Wolbachia coinfections extend to the entire phage genome. Below, we provide evidence of a full bacteriophage transfer between these two coinfecting Wolbachia.

Studying the genomes of fastidious bacteria that cannot be cultured outside of their eukaryotic host cells often presents practical problems owing to a difficulty in sequencing a genome that might be a small minority of the total DNA in a host. In addition, the amplification methods that have been used to overcome small DNA yields can introduce chimeras and mutations in the resulting sequence. Below we describe the first use of a high-density tiled oligonucleotide array for efficient enrichment and sequencing of this endosymbiont’s DNA. Based on the results, we propose that targeted genome capture can be widely used to isolate the small genomes of symbiotic members of eukaryotic hosts.
Materials and Methods

Sequence Capture Array

The targeted sequence capture array of 385,000 tiled probes was designed by Roche NimbleGen from whole genome sequences and shotgun sequences from the Wolbachia infections of Culex pipiens Pel (NC_010981), Drosophila melanogaster yw (NC_002978), Drosophila simulans Riverside (NC_012416), Brugia malayi (NC_006833), Drosophila ananassae (NZ_AAGB00000000), Muscidifurax uniraptor (NZ_ACFP00000000), and Drosophila willistoni (NZ_AAQP00000000) and prophage sequences from the Wolbachia infections of Cadra cautella (AB161975.2, AB478515.1, AB478516.1) and Ephesia kuehniella (AB036666.1). Sequences also present in the insect genomes of Aedes aegypti (AAGE02000000), Apis mellifera (NC_007070–NC_007085), D. melanogaster (NC_004353, NC_004354, NS_000188, NT_033777, NT_033778, NT_03379, NT_037436), Drosophila sechellia (AAKO01000000), Tribolium castaneum (NC_007416–NC_007425), N. vitripen-

Fig. 1.—Gene presence and synteny between prophages WOVitB and WOVitA1. WO genes are syntenous and homologous between prophages WOVitB and WOVitA1. WOVitB genes contained within a single contig are denoted with an underline. WOVitB reads mapped to repetitive flanking regions of WOVitA1 are denoted with a dashed line. The other genes category includes genes annotated as a holliday junction resolvase, a leucine-rich repeat protein, DNA polymerase III, an ATPase, and a methyl accepting chemotaxis protein.
rmap package, version 2.0.5 (Smith et al. 2009). This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AERW00000000. The version described in this paper is the first version, AERW01000000. Sequences for phages WOVitA1 and WOVitB have been deposited under accession numbers HQ906662–HQ906666.

Comparison of the wVitB and wVitA Phage Regions

Coding sequences for phage WOVitA1, WOVitA2, and WOVitA4 and the 5' and 3' flanking regions from the genome project for wVitA (M.C., I.L.G.N., S. Richards, B.N.K., S.R.B; and J. Werren) were identified using Blast. WOVitA1 ORFs (supplementary table S1, Supplementary Material online) used in this analysis were assigned using the Geneious ORF finder algorithm as described for wVitB. These coding sequences were searched against all wVitB contigs using MegaBlast to identify phage regions and determine percent homology between homologs. Raw reads were also assembled to WOVitA1 and its flanking regions to identify contiguous segments of WOVitB sequence. Oligonucleotide primers were designed so that polymerase chain reaction (PCR) could be used to assemble the phage contigs into a full-length phage where possible. 2) determine if phage genes that were not full-length when compared with their homologs in WOVitA1 were truly possible, and 2) determine if phage genes that were not full-length were searched against all wVitB sequence. Oligonucleotide primers were designed so that polymerase chain reaction (PCR) could be used to assemble the phage contigs into a full-length phage where possible, 2) determine if phage genes that were not full-length when compared with their homologs in WOVitA1 were truly missing these sequences in the wVitB genome, and 3) determine if phage genes found in WOVitA1 but not in WOVitB were absent in the wVitB sequence (supplementary tables S1 and S7, Supplementary Material online). PCR was performed using the Phusion High-Fidelity enzyme kit (New England Biolabs) with 25 ng of genomic DNA from N. vitripennis WPa_0411–0455, and WPa_1294–1340.

Results

Phage Genome Transfer

We array captured and sequenced the genome of the Wolbachia wVitB, which was previously segregated from its natural wVitA coinfection in N. vitripennis (Perrot-Minnot et al. 1996). Wolbachia wVitB is a low titer infection at 0.01 Wolbachia gene copies/1 Nasonia gene copy (Bordenstein et al. 2006), which extrapolates to approximately 25,000 Nasonia base pairs per one Wolbachia base pair.

Array capture and subsequent Illumina sequencing indicated a single bacteriophage WO haplotype, WOVitB, in wVitB. This single haplotype was compared with the three WOVitA prophages in the A-Wolbachia to determine if a complete phage WO transfer occurred between wVitA and wVitB (fig. 1). Seven contigs from the wVitB assembly were originally syntenous to sequences from WOVitA1. Subsequent mapping of raw Illumina reads to the WOVitA1 genome, PCR, and Sanger sequencing were employed to complete the WOVitB phage sequence in two contigs. Sixty-nine percent (35/51) of WOVitA1 genes were present in WOVitB (supplementary table S1, Supplementary Material online). Of these 35 genes, five WOVitB genes are not full coding length. The remaining genes in phase WOVitA1 have been deleted from WOVitB. In two cases, a 5 kb deletion in the head region and a 4.5 kb deletion in the tail region, genes have been replaced by transposon insertions of the ISSod13 family (fig. 1, supplementary table S1, Supplementary Material online). Inability to PCR-amplify genes that were absent in WOVitB, but present in WOVitA1, confirmed their loss from the wVitB genome.

The 35 prophage coding sequences and partial genes of the WOVitB genome are, on average, 99.9% similar to genes in phage WOVitA1 (range 98.7–100%) (fig. 2). This nucleotide identity is significantly higher than the 83.3% average nucleotide identity of the remaining phage genes in the wVitB genome (range 68.4–89.8%) (supplementary table S2, Supplementary Material online) that are not related to the transferred WOVitA1 (fig. 2, Mann–Whitney U, two-tailed, P < 0.0001). These genes are homologous to...
WoVitA1 is provided in supplementary table S2 (Supplementary Material online). A list of phage gene homologs present in WoVitA and WoVitB previously sequenced protein-coding genes. A list of phage gene homologs present in WoVitB that are not found in WoVitA1 is provided in supplementary table S2 (Supplementary Material online).

The truncated tail region of WoVitB is adjacent to the same location as the tail region of WoVitA1, which contains helix-turn-helix transcriptional regulators, the gene encoding the DNA repair protein RadC, the gene mutL, a gene encoding a heat shock protein, conserved hypothetical genes, and ankyrin-repeat proteins (fig. 1). Many of these genes are involved in DNA binding or protein–protein interactions that could regulate the lytic or lysogenic state of the phage (Kolkhof et al. 1992; Tourasse and Kolsto 2008). Like the defined phage region, the homology between WoVitA and WoVitB is also nearly identical in these genes, indicating that they are part of the transferred phage. Interestingly, Wo phages from strains wPip (Klasson et al. 2008), wMel (Wu et al. 2004), wCauB (Tanaka et al. 2009), and wRi (Klasson et al. 2009) are also integrated near some of these same genes, signifying that integration of some Wo types may be preferential for this region or this region is part of the Wo phage. Taken together, these findings indicate a recent whole genome transfer event followed by some erosion due to transposon insertions and gene loss.

Efficiency of Targeted Genome Capture

Illumina sequencing of the captured DNA yielded 5.04 million paired-end reads that were assembled and manually edited. The final assembly was comprised of 426 contigs (≥100 bp) totaling 1,107,643 bp. Only 1.7% of the assembled nucleotides before manual editing had non-Wolbachia sequence matches (fig. 3). Fifty-four contigs were from the host N. vitripennis (supplementary table S3, Supplementary Material online) and 28 contigs were from other bacteria such as Proteus, Arsenophonus, Providencia, and Xenorhabdus (supplementary table S4, Supplementary Material online), which are known infections of Nasonia (Werren et al. 2010). Forty-four contigs spanning 0.6% of the assembly had no significant match in GenBank, yet their average GC content was 36%, similar to the typical GC content in

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Wolbachia genomes. These contigs likely represent novel sequences in the wVitB genome that were array captured by either adjacent regions to hybridized DNA fragments or sequential hybridization of captured DNA acting as template for flanking DNA not printed on the array.

The above analyses indicate that the array capture was highly specific to the bacterial endosymbiont. Next we tested the completeness of the captured genome. We note five observations:

1) First, the assembly has 34 tRNAs, one 16S, 23S, and 5S rRNA gene, and a 34% GC content that typify all Wolbachia genomes thus far (Wu et al. 2004; Klasson et al. 2008; Klasson et al. 2009).

2) Second, of the 756 core genes present in four fully sequenced Wolbachia genomes, only 13 were absent in the captured wVitB, including a drug resistance transporter, genes of a phage HK97 family remnant not related to phage WO, and hypothetical proteins (supplementary table S5, Supplementary Material online). Absent genes from the core genome were compared with the array sequence to ensure that these sequences were available on the array for pull-down, and all 13 sequences were present.

3) Third, 81.6% of genes from the B-Wolbachia wPip of Culex quinquefasciatus Pel, the closest sequenced relative to wVitB, were present in wVitB (supplementary fig. S2, Supplementary Material online). The remaining wPip genes that did not have homologs in the wVitB sequence (supplementary fig. S3, Supplementary Material online), included 55 phage genes from the five phage types present in wPip, 56 transposases, and 30 ankyrin-repeat proteins, all of which have been shown to be highly variable between Wolbachia sequences (Wu et al. 2004; Iturbe-Ormaetxe et al. 2005; Klasson et al. 2008). A high number (76) of the additional absent genes are of unknown function. A full list of the wPip genes lacking orthologs in the pulled-down wVitB sequence is provided in supplementary table S6 (Supplementary Material online).

4) Fourth, eight previously sequenced protein-coding genes (Baldo et al. 2006; Paraskevopoulos et al. 2006) from wVitB are identical to the same genes in the captured wVitB sequence. In addition, the average nucleotide identity between the wVitB genes captured and wPip is 95.7% and the lowest nucleotide similarity between the wVitB sequence and the array sequence is 78.5% (316 bp of a transposase sequence), indicating the array methodology is sufficient to pull-down sequences of closely related strains, species, or even genera.

5) Finally, the distributions of the length and coverage of the assembled contigs are consistent with the distributions obtained by assembling the same number and type of paired-end Illumina reads simulated from the genome of the Wolbachia endosymbiont of Culex quinquefasciatus Pel (Klasson et al. 2008) (fig. 4). However, a sharp peak was observed in the simulated data at 15× coverage.
whereas the array coverage data was more evenly distributed, likely due to the efficiency of the array capture. These data suggest that the capture is comprehensive, and that any genome fragments missing are due to either their true absence from the genomes or a failure to assemble them, rather than failure to capture them. The 1.1 Mb genome size is mostly consistent with previously sequenced Wolbachia genomes, including the Wolbachia wMel strain that is 1.27 Mb (Wu et al. 2004; Klasson et al. 2008; Klasson et al. 2009; Scott and Ghedin 2009).

**Discussion**

The implication of frequent mobile element transfers between obligate intracellular coinfections is significant to genome evolution in host-associated microbes. If eukaryotic hosts can be arenas for HGT between intracellular infections, especially those bacteria that frequently move between different hosts (Bordenstein and Reznikoff 2005; Kent and Bordenstein 2010), then these bacteria can partially ease the reductive processes of an intracellular lifestyle. Indeed, minor capsid gene sequences are identical between Wolbachia coinfections in every examined species thus far (Masui et al. 2001; Bordenstein and Wernegreen 2004; Chafee et al. 2010), implying that bacteriophage transfer between Wolbachia coinfections in the same host is likely the rule rather than the exception. However, evidence of phage transfer outside of this single gene had not previously been shown.

**Horizontal Transfer of WO**

Here, we demonstrated for the first time that large-scale HGT (52.2 kb) of phage and flanking genes occurred between the wVitA and the wVitB coinfections of N. vitripennis. The transferred haplotypes, WOVitB and WOVitA1, are distinct from the WO haplotypes found in other Wolbachia in both synteny and nucleotide identity (data not shown), demonstrating that the exchange happened within the wasp host. Complete and frequent transfer of temperate bacteriophage WO likely supports its spread in the Wolbachia genome. Transfer of prophage WO genomes in other Wolbachia coinfections is common, and it remains to be determined why some Wolbachia genomes maintain intact phages, whereas others exhibit remnants of the phage genome.

Other examples of mobile element transfers larger than insertion sequences are restricted to just two cases—both in other widespread obligate intracellular bacteria. In Rickettsia, genome-sequencing projects have identified the transfer of a 54.6-kb ICE between a relative of Rickettsia bellii and Rickettsia massiliae (Blanc et al. 2007). In addition, clusters of Rickettsial plasmid conjugal transfer genes were found in the genome, which could indicate horizontal transfer of the plasmid followed by genome integration. Within Chlamydia pneumoniae, in vitro cocultivation experiments with different strains that were positive and negative for a small lytic bacteriophage family (4,524 bp) showed that bacteriophages can be transmissible between cells in the laboratory (Rupp et al. 2007).

**Targeted Genome Capture of Microbial Symbionts**

By array capturing the genome of an organism that lives within cells of a host carrying other bacterial species and viruses, we demonstrated that the “targeted genome capture” method is highly efficient for enriching and sequencing specific microorganisms from a heterogeneous mixture of DNA. Since this method relies on sorting DNA and not cells or tissues, it could be applied to samples that have been fixed or frozen. We propose that this method can be widely applied to other bacterial and viral symbiont genomes, including human pathogens that exist in low titers.
However, some caution is required. First, our results show capture arrays unexpectedly have the capacity to enrich for divergent sequences, with as much as 78.5% nucleotide divergence. The risk is that multiple strains or species within the same host could be captured together; whereas the benefit is that the capture is not restricted to closely related strains. If multiple haplotypes are captured on the same array, binning heterologous DNA sequences bioinformatically could circumvent problems associated with assembling various haplotypes. Second, the extent of captured DNA can be constrained by the range of probes printed on the array, such that novel genomic sequences within the target genome may be problematic to capture. However, our results show that 0.6% of the nucleotides in the final genome are potentially new Wolbachia sequences based on their low GC content typical of the endosymbiont. We hypothesize that captured genes could act as single-stranded probes for the subsequent capture of genes not printed on the array.

Genomic analyses of fastidious symbionts that are difficult to culture or purify from their hosts often rely on isolations from sensitive host tissue collections, whole genome amplifications that subject the template DNA to artificial chimeras, or are not possible. Targeted genome capture has the potential to obviate these pitfalls for rapid enrichment and sequencing of organisms with relatively small genomes.

In summary, we have found that transfer of bacteriophage between coinfections of obligate intracellular bacteria in the same host can span entire phage genomes with many genes and that targeted sequence capture can trap genomes for de novo sequencing of new microbial or viral symbionts from eukaryotic hosts. Although the array capture method intrinsically depends on preexisting sequence information, we have shown that the captured sequences can be divergent from the arrayed DNA. The method offers significant improvements over labor-intensive alternatives for sequencing symbionts that are difficult to purify or culture.

Supplementary Material
Supplementary data sets S1–S16, figures S1–S3, and tables S1–S7 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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