Extensive genomic diversity of closely related *Wolbachia* strains

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Using microarray-based comparative genome hybridization (mCGH), the genomic content of *Wolbachia pipientis* wMel from *Drosophila melanogaster* was compared to the closely related *Wolbachia* from *D. innubila* (wInn), *D. santomea* (wSan), and three strains from *D. simulans* (wAu, wRi, wSim). A large number of auxiliary genes are identified in these five strains, with most absent/divergent genes being unique to a given strain. Each strain caused an average of ~60 genes to be removed from the core genome. As such, these organisms do not appear to have the streamlined genomes expected of obligate intracellular bacteria. Prophage, hypothetical and ankyrin repeat genes are over-represented in the absent/divergent genes, with 21–87 % of absent/divergent genes coming from prophage regions. The only wMel region absent/divergent in all five query strains is that containing WD_0509 to WD_0511, including a DNA mismatch repair protein MutL-2, a degenerate RNase, and a conserved hypothetical protein. A region flanked by the two portions of the WO-B prophage in wMel is found in four of the five *Wolbachia* strains as well as on a plasmid of a rickettsial endosymbiont of *Ixodes scapularis*, suggesting lateral gene transfer between these two obligate intracellular species. Overall, these insect-associated *Wolbachia* have highly mosaic genomes, with lateral gene transfer playing an important role in their diversity and evolution.

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INTRODUCTION

Wolbachia bacteria are common obligate intracellular endosymbionts that infect a wide variety of invertebrates,

including arthropods and filarial nematodes. The arthropod-infecting *Wolbachia* strains are maternally inherited and exert unusual effects on host reproduction, including: (1) parthenogenesis, whereby infected virgin females produce infected female offspring, (2) male killing, whereby infected male embryos fail to develop, (3) feminization, whereby genetic males develop into reproductively capable females, and (4) cytoplasmic incompatibility, the most common phenotype, whereby the offspring of uninfected females and infected males fail to develop (Stouthamer *et al.*, 1999; Werren *et al.*, 2008). These phenotypes increase the number of infected hosts within a population, promoting the frequency of *Wolbachia*. Although insect-associated *Wolbachia* are typically considered reproductive parasites, some

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Wolbachia strains have no reproductive phenotype (Hoffmann *et al.*, 1996; Turelli & Hoffmann, 1995), and mutualistic host–bacteria interactions have also been described (e.g. Dobson *et al.*, 2002; Hedges *et al.*, 2008; Teixeira *et al.*, 2008). Phylogenetic analysis of concatenated protein sequences and the 16S rRNA places *Wolbachia* in a clade of obligate intracellular bacteria including the tick- and trematode-vectored vertebrate pathogens from the genera *Anaplasma*, *Ehrlichia* and *Neorickettsia*, as well as the more distantly related, but better characterized, arthropod-associated *Rickettsia* spp. (Dumler *et al.*, 2001; Dunning Hotopp *et al.*, 2006b). Numerous phylogenetic analyses of *Wolbachia* have been undertaken, including single-locus and multi-locus approaches (Bordenstein *et al.*, 2008; Casiraghi *et al.*, 2005; Lo *et al.*, 2002; O'Neill *et al.*, 1992; Werren *et al.*, 1995; Zhou *et al.*, 1998). Despite maternal inheritance in arthropods, the phylogenetic distribution of these *Wolbachia* is not concordant with that of their hosts (Baldo *et al.*, 2006b; O'Neill *et al.*, 1992; Werren *et al.*, 1995). Therefore, in arthropods both horizontal transmission of *Wolbachia* and loss of infection are likely, although the mechanisms are not fully understood (Bouchon *et al.*, 1998; Heath *et al.*, 1999; Huigens *et al.*, 2004).

Species designations in *Wolbachia* have remained elusive, largely owing to the lack of an accepted definition of bacterial species that can be applied to obligate intracellular bacteria. However, current phylogenetic and recombination analysis supports all *Wolbachia* strains being formally included in the single species *Wolbachia pipientis* (Lo *et al.*, 2007). To help distinguish isolates, *Wolbachia* strains have been divided into eight phylogenetic clades termed supergroups (Bordenstein *et al.*, 2008; Lo *et al.*, 2007). The A and B supergroup *Wolbachia* are the most common and are widespread in arthropods as single or double infections (Werren *et al.*, 1995). The C and D supergroups contain the *Wolbachia* strains from filarial nematodes (Lo *et al.*, 2002). Primitive wingless insects (springtails; Collembola) are infected with supergroup E (Czarnetzki & Tebbe, 2004; Lo *et al.*, 2002; Vandekerckhove *et al.*, 1999). Supergroup F consists of *Wolbachia* infecting termites, weevils, true bugs and filarial nematodes (Casiraghi *et al.*, 2001; Lo *et al.*, 2002; Rasgon & Scott, 2004). Supergroup H infects termites (Bordenstein & Rosengaus, 2005). Three other divergent lineages exist that have not been given supergroup designations, including the infection found in the flea *Ctenocephalides canis* (Casiraghi *et al.*, 2004), the filarial nematode *Dipetalonema gracile* (Casiraghi *et al.*, 2004), and the pseudoscorpion *Cordylochernes scorpioides* (Zeh *et al.*, 2005; Zeh & Zeh, 2006). Double or triple infections have been described with isolates coming from different or identical supergroups (Werren *et al.*, 1995).

Obligate intracellular bacteria are often viewed as excellent model systems to understand the stable, symbiotic interactions that occur between prokaryotes and their eukaryotic hosts. However, recent analyses of sequenced genomes of host-switching obligate intracellular bacteria,

like *Wolbachia*, suggest that mobile elements and genetic exchange are not as rare as previously thought (Bordenstein & Reznikoff, 2005). The presence of extensive recombination (Baldo *et al.*, 2006a; Klasson *et al.*, 2009), mobile elements (Klasson *et al.*, 2008; Wu *et al.*, 2004) and lateral phage transfer between co-infecting strains (Bordenstein & Wernegreen, 2004; Masui *et al.*, 2000) facilitates high levels of genetic exchange in *Wolbachia*. These processes that shape the *Wolbachia* genome are likely influenced by exposure to novel gene pools available due to horizontal transmission of infection, multiple infections and large numbers of infected hosts.

The high level of genetic exchange in *Wolbachia* suggests that like many free-living bacteria, *Wolbachia* has a core genome complemented by an extensive auxiliary genome. A core genome consists of all the genes shared by all (or almost all) sequenced strains for a given taxon, typically a species (Boucher *et al.*, 2001; Hogg *et al.*, 2007; Lan & Reeves, 2000; Tettelin *et al.*, 2005, 2008). All of the other genes constitute what has been termed the dispensable (Tettelin *et al.*, 2005, 2008), contingency (Hogg *et al.*, 2007) or auxiliary (Boucher *et al.*, 2001; Lan & Reeves, 2000) genome, which encompasses the genetic variation within the species. A subset of the auxiliary genes includes the unique (Hogg *et al.*, 2007) or strain-specific genes (Tettelin *et al.*, 2005, 2008), those genes found only in a single genome of the species. When the auxiliary genome is combined with the core genome, the result is the pan-genome (Tettelin *et al.*, 2005, 2008) or supragenome (Hogg *et al.*, 2007). Pan-genomes can be classed as open/unbounded or closed/bounded (Medini *et al.*, 2005; Tettelin *et al.*, 2008). With an open pan-genome, each additional genome sequenced, on average, contributes new genes to the pan-genome. In contrast, with a closed pan-genome, each additional genome sequenced contributes no new genes to the pan-genome. Species with open pan-genomes include *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Escherichia coli*, *Bacillus cereus* and *Prochlorococcus marinus* (Tettelin *et al.*, 2008). Those with closed pan-genomes include *Staphylococcus aureus*, *Streptococcus pyogenes*, *Ureaplasma urealyticum* and *Bacillus anthracis* (Tettelin *et al.*, 2008). In addition to the above nomenclature, another convention has been proposed that combines all genes found in >1 % of strains into a species genome, and any genes in <1 % of strains are considered of foreign origin or on the decline (Boucher *et al.*, 2001; Lan & Reeves, 2000).

In order to examine the core genome and the genetic flux between *W. pipientis* strains, we examined the genomic content of five strains using microarray-based comparative genomic hybridization (mCGH) on a *Wolbachia* microarray. DNAs from reference and query organisms are differentially labelled and competitively hybridized to a microarray (Behr *et al.*, 1999). The hybridization data reveal the presence/absence of genes in the query genome relative to the genome content of the reference organism. For *Wolbachia*, a large number of auxiliary genes are

identified in five closely related supergroup A strains, with most absent/divergent genes unique to a given strain. Prophage, hypothetical and ankyrin repeat genes are over-represented in the absent/divergent genes, with 3–55 of those being unique to a given strain. One variable region flanked by the two portions of the WO-B prophage in *wMel* is also identified in a plasmid of a *Rickettsia* endosymbiont of *Ixodes scapularis* (Noda *et al.*, 1997) suggesting that lateral gene transfer extends across the order Rickettsiales.

METHODS

Bacterial strains. Genomic DNA from six *Wolbachia* strains was hybridized in this study (Table 1). These are a subset of well-studied supergroup A strains that were chosen because they were expected to be genetically similar but induce a variety of reproductive phenotypes. The supergroup A *Wolbachia* strains from *Nasonia vitripennis* 12.1, *Drosophila recens*, *D. yakuba* and *D. teissieri* were also initially chosen for inclusion, but were later removed because of difficulties in background hybridization, likely due to host DNA contamination.

Microarray construction. A *wMel* microarray was constructed which represents 95 % of the unique ORFs (Wu *et al.*, 2004). Primers were designed from the unique predicted genes annotated in *wMel*. In order to obtain pure amplicons devoid of background amplification of host genes, all amplicons were produced, where possible, from two separate clones from the *wMel* genome-sequencing project. Genes in physical gaps where a sequencing clone was not available were amplified directly from genomic DNA from *Drosophila melanogaster* infected with *wMel*. Amplicons (50–1000 bp) were produced using AmpliTaq (Applied Biosystems), purified using filtration plates (Millipore), and analysed on agarose gels. The purified amplicons were diluted 1:1 with DMSO, spotted in quadruplicate onto Corning UltraGap slides with a Lucidea printing robot (GE Healthcare) and irradiated with UV light (Dunning Hotopp *et al.*, 2006a).

Genomic DNA preparation and whole-genome amplification. *Wolbachia* cells were isolated from 2–4 g of live, whole insects via density centrifugation, and whole chromosomal fragments were purified following pulsed-field gel separation as described by Wernegreen *et al.* (2002). Due to the small amounts of chromosomal fragments recovered, whole-genome amplification was needed to produce enough DNA for hybridization. Therefore ~10 ng of genomic DNA was amplified per reaction with GenomiPhi. A similar strategy was used successfully for mCGH studies in *Anaplasma phagocytophilum* (Dunning Hotopp *et al.*, 2006b). Whole-genome amplification has been shown to be specific, with a randomly distributed bias and a mutation rate of $<2 \times 10^{-5}$ (Zhang *et al.*,

2006). In the obligate intracellular bacterium *Ehrlichia chaffeensis* Arkansas, a comparison of genomic DNA with GenomiPhi-amplified DNA also revealed a randomly distributed bias (data not shown). A similar comparison of genomic DNA to GenomiPhi-amplified DNA was not possible for *Wolbachia* DNA in the current study due to an inability to obtain large quantities of genomic DNA. However, a comparison of two independent GenomiPhi amplifications of the same genomic DNA sample from strain *wRi* demonstrates the reproducibility of the amplification (Fig. 1). To reduce the effects of any bias, each probe was synthesized from a pool of three distinct GenomiPhi amplifications.

Microarray hybridizations. Cy3 and Cy5 probes were synthesized from GenomiPhi-amplified DNAs as described by Tettelin *et al.* (2001). Briefly, amino-allyl-dUTP-labelled probes were synthesized from 4 µg of genomic DNA using Klenow fragment (3'→5' exo-) (New England Biolabs). The reactions were purified using the QIAquick PCR purification kit (Qiagen) with modified phosphate buffers. Cy3 or Cy5 dyes (GE Healthcare) were chemically coupled to the incorporated amino-allyl-dUTP in carbonate buffer. Probe pairs were resuspended in $5 \times$ SSC, 50 % formamide, 0.1 % SDS, 0.1 mM DTT and 0.35 mg ml^{-1} salmon sperm DNA and hybridized to slides overnight at 42 °C. The hybridized slides were washed twice in $2 \times$ SSC, 0.1 % SDS at 55 °C; twice in $0.1 \times$ SSC, 0.1 % SDS at room temperature; twice in $0.1 \times$ SSC at room temperature; and once in MilliQ water at room temperature. They were then dried, and scanned using a GenePix4000B scanner (Molecular Devices).

Microarray data analysis and bioinformatics. Cy3- and Cy5-labelled probes were synthesized from a pool of three distinct GenomiPhi amplifications to reduce bias. One flip-dye experiment (two hybridizations) was performed with each of three independent pools, yielding a total of six hybridizations per strain. With 4–8 printed replicates per slide this yielded 24–48 replicated spots per gene to compare across the study. Ratios were calculated as *wMel* reference fluorescence over the query fluorescence (reference/query) and were normalized using iterative log-mode centring (Dunning Hotopp *et al.*, 2006a). The geometric mean ratio was calculated for all good spots in each flip-dye experiment. The values reported are the medians of the geometric mean ratio for the three flip-dye experiments. Ideally, hybridization results would be compared to gene presence/absence from two closed genome sequences to establish microarray platform-specific criteria for absence/divergence. However, only one supergroup A *Wolbachia* has had its genome fully sequenced and closed. Therefore, previously established criteria for a *Neisseria meningitidis* microarray were used (Dunning Hotopp *et al.*, 2006a), with genes deemed absent/divergent (ratio >3), present (ratio <2), or not predictive (ratio 2–3).

Southern hybridizations. Total genomic DNA from *Drosophila* lines was extracted according to the Holmes–Boner method (Sambrook *et al.*, 1989). The DNA fragments generated from 5 µg

Table 1. *Wolbachia* strains used in this study

Strain	Host	Reproductive phenotype	Reference
<i>wMel</i>	<i>Drosophila melanogaster</i>	Cytoplasmic incompatibility	Wu <i>et al.</i> (2004)
<i>wInn</i>	<i>Drosophila innubila</i>	Male killer	Dyer & Jaenike (2005)
<i>wAu</i>	<i>Drosophila simulans</i>	None	Hoffmann <i>et al.</i> (1996); Turelli & Hoffmann (1995)
<i>wRi</i>	<i>Drosophila simulans</i>	Cytoplasmic incompatibility	Hoffmann <i>et al.</i> (1986)
<i>wSim</i>	<i>Drosophila simulans</i>	Unknown	(Salzberg <i>et al.</i> (2005)
<i>wSan</i>	<i>Drosophila santomea</i>	Cytoplasmic incompatibility	Zabalou <i>et al.</i> (2004); Zabalou <i>et al.</i> (2008)

Fig. 1. Comparison of independent hybridizations of DNA from whole-genome amplifications. The ratios from two flip-dye experiments for strain *w*Ri are positively correlated, with $R^2=0.73145$, indicating the reproducibility of whole-genome amplification of the samples.

*Eco*RI-digested genomic DNA were separated on a 0.8 % agarose gel and blotted onto Immobilon-Ny+ filter (Millipore) according to the manufacturer's instructions and hybridized at 68 °C for 18 h. WD_0514 ANK probe was generated by PCR amplification with Aorf818F 5'-ATACAGTGGCCGATAAATAAC-3' and Aorf818R 5'-AGCACCAACAATAAACTTCTCA-3' in 50 µl reactions containing 50 mM KCl, 10 mM Tris/HCl pH 9.0, 0.1 % Triton X-100, 1.5 mM MgCl₂, 125 µM dNTPs, 500 nM of each primer, 1 U Taq DNA polymerase (Promega) and 50 ng DNA, with an initial denaturation step at 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 3 min; and a final elongation step at 72 °C for 10 min. PCR products were purified with a PCR purification kit (Qiagen) and radioactively labelled with [α -³²P]dATP using the Prime-a-gene labelling kit (Promega). A specific probe for the *Wolbachia* *ftsZ* cell-division gene was also generated with the primers *ftsZ*1 5'-GTATGCCGATTGACAGAGCTTG-3' and *ftsZ*2 5'-GCCATGAGTATTCACCTTGGCT-3' (Holden *et al.*, 1993) and used as positive control for *Wolbachia* DNA.

PCR amplification. MLST profiles of *w*San and *w*Sim were obtained as previously described (Baldo *et al.*, 2006b) using Taq (Promega) or HotStarTaq (Qiagen). The region from WD_0509 to WD_0514 was amplified using primers 467F (5'-TTCTTGATCTCACAATCTCTA-3') and 566R (5'-AGACTGTGCCTGAGAATCAA-3') with a 98 °C initial denaturation for 30 s; 35 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 20 min; and a final 72 °C elongation for 10 min using either Elongase (Invitrogen) or Phusion (Finnzymes) according to the manufacturer's instructions with 1.5 mM MgCl₂ and 2 mM dNTPs. PCR products were purified with a PCR purification kit (Qiagen) or treated with shrimp alkaline phosphatase and exonuclease I (Amersham). Sequencing was performed by Macrogen and the Institute for Genome Sciences at the University of Maryland Baltimore.

RESULTS

Core genome

The core bacterial genome can be defined as those genes conserved among all bacterial strains of a given species. In

this study, each query strain leads to the removal of ~60 genes on average from the core *W. pipientis* genome (Fig. 2B). An exponential regression suggests that the core genome consists of 621 genes. Although this estimate is based on only five data points, it is roughly consistent with the 743 *w*Mel genes in the 637 shared clusters of orthologous gene clusters between *w*Mel and the most distantly related sequenced supergroup D *Wolbachia* genome, *w*Bm, as described in the Rickettsiales Comparative Database (Dunning Hotopp *et al.*, 2006b). The similarity of these numbers is somewhat surprising considering the similarity of the five strains compared by mCGH when compared to the divergence of *w*Bm. In addition, the microarray-defined core genome uses high-stringency DNA identity as opposed to the bi-directional reciprocal best protein matches for the orthologue prediction. Despite these differences, 597 *w*Mel proteins are present in both the orthologue prediction and the microarray-defined core genome. The convergence of these two core genome metrics across diverse strains may suggest a single *Wolbachia* core genome that spans the supergroup designations, thus supporting the single species designation of *W. pipientis* (Lo *et al.*, 2007).

Mobile genetic elements

Bacteriophage and other mobile elements are notable catalysts of lateral gene transfer and traditionally have been considered absent in the genomes of obligate intracellular bacteria. However, in species that experience horizontal transmission to new hosts, moderate to high levels of mobile genetic elements have been observed (Bordenstein & Reznikoff, 2005; Wu *et al.*, 2004). Previous studies of *Wolbachia* phage indicate rampant lateral phage transfer between divergent strains and that the mechanism of transfer is in part mediated via the high rate of *Wolbachia* co-infections (Bordenstein & Wernegreen, 2004; Masui *et al.*, 2000). Mobility of *Wolbachia* phage could promote the introduction of novel genes by shuttling large fragments of *Wolbachia* DNA between genomes.

The mCGH results show that phage genes are significantly over-represented in the absent/divergent genes (Table 2), supporting the idea that these strains with similar genomic backbones are actively exchanging phage regions. Of the absent/divergent genes identified, 21–87 % are in the prophage regions (Fig. 3). Although all of the strains examined here have portions of each prophage (Fig. 4B), each also appears to be missing sections of these regions.

One region of interest is the region encompassing WD_0612–WD_0618 that is flanked by portions of WO-B. It encodes an NAD-dependent epimerase/dehydratase family protein, a glycosyltransferase with a radical SAM domain, two putative L-allo-threonine aldolases, an ABC transporter permease and ATP-binding protein, a conserved domain protein, and a conserved hypothetical protein (Fig. 4A). This region was conserved in all but *w*Inn in the current study (Fig. 4B), but was not identified

Fig. 2. Genomic diversity and the core genome. (A) Most gene differences are unique to a strain, resulting in different sets of absent/divergent genes (ratios ≥ 3) per strain, with only three genes absent/divergent in all five strains. Numbers within the intersections of different ovals count the number of absent/divergent genes (ratio ≥ 3) shared by 2, 3, 4 or 5 organisms. (B) The number of *Wolbachia* core genes for n query genomes is plotted against the number of query genomes compared. For example, when $n=3$, each query genome is compared to all possible combinations of two other query genomes to see how many genes are shared between the three. Each combination ($\blacklozenge$), the mean of each combination ($\blacksquare$), and the standard deviation (error bars) are presented. With the addition of each genome a significant number of genes are removed from the core genome, demonstrating that even between these closely related *Wolbachia* strains there is a sizeable amount of genomic diversity. The nonlinear regression of $620.9 + 557.9e^{-0.204x}$ (RSS=0.0148) suggests that the core genome consists of 621 genes.

Table 2. TIGR role categories of overrepresented absent/divergent genes

Fisher's Exact test, Bonferroni stepdown correction; $P < 0.01$.

Strain	TIGR role category (P value)
<i>Drosophila simulans</i> wAu	Hypothetical proteins (1.3×10^{-17})
<i>Drosophila innubila</i> wInn	Hypothetical proteins (8.0×10^{-3})
<i>Drosophila simulans</i> wRi	Hypothetical proteins (5.2×10^{-6})
	Prophage functions (5.6×10^{-4})
<i>Drosophila simulans</i> wSim	Prophage functions (9.3×10^{-3})
<i>Drosophila simulans</i> wSan	Prophage functions (2.7×10^{-10})
Combined (all five strains)	Hypothetical proteins (3.9×10^{-26})
	Prophage functions (1.0×10^{-4})

in either the wBm or wPip genome sequences. Interestingly, this same region was uncovered in a bacterial plasmid in the genome sequencing of *I. scapularis* (GenBank ABJB000000000) and is attributed to a concomitantly sequenced ovary-associated *Rickettsia* endosymbiont (Noda *et al.*, 1997; Steiner *et al.*, 2008). This endosymbiont is most closely related to other tick-associated *Rickettsia* spp. (Weinert *et al.*, 2009). The plasmid is not thought to arise from a lower-level contaminating *Wolbachia* infection because (a) only 44 *Wolbachia* sequences limited to this ~10 kbp region were recovered in the whole-genome shotgun sequencing project (Dunning Hotopp *et al.*, 2007) as opposed to the 100 000+ reads recovered with homology to the rickettsial endosymbiont; (b) the 16S rRNA and *wsp* genes could not be amplified from the *I. scapularis* genomic DNA with *Wolbachia*-specific primers; and (c) of the 40 non-transposase ORFs on the plasmid, 23 have a rickettsial best match (e-value $< 10^{-15}$), including genes involved in plasmid maintenance, while only the seven corresponding to WD_0612–WD_0618 have a *Wolbachia* best match (e-value $< 10^{-100}$). Of the remaining non-transposase ORFs on the plasmid, eight have a *Lawsonia intracellularis* best match (e-value $< 10^{-50}$), and two have a best match to other bacteria.

In the *Rickettsia* endosymbiont plasmid, this ~10 kbp region is flanked by transposases (Fig. 4A). The lack of divergence (~70 % nt identity) and association with mobile elements strongly suggests lateral gene transfer between these micro-organisms. No closely related orthologues are in public databases to facilitate assigning the direction of this transfer or determining whether a third, intermediate organism was involved. Regardless, this is a compelling case of lateral gene transfer between intracellular bacteria of different genera.

As an additional example of mobile element transfer between intracellular bacteria of diverse genera, *Wolbachia* and the unrelated bacterial agent of cat-scratch disease, *Bartonella henselae*, share divergent prophage homologues of prophage WO-B (Alsmark *et al.*, 2004). These two intracellular parasites likely exchanged phage genes in the

Fig. 4. Prophage regions. Four prophage regions are compared, including the canonical *wKue* WO phage, the two *wMel* WO prophage, and a representative prophage from *wPip* (A). Absent/divergent genes (red; ratio >3), ambiguous genes (blue; ratio 2–3) and possible duplicated genes (green; ratio <0.3) are shown under the representative *wMel* prophage region (B). The prophage regions appear to have a high level of variation. A region flanked by *wMel*'s WO-B is homologous to a plasmid sequenced from a rickettsial endosymbiont of *I. scapularis* (A). This region appears to be the result of lateral gene transfer between *Wolbachia* and *Rickettsia*. It is present in *wAu*, *wSim*, *wRi* and *wSan* in the current study, but not in the genomes of *wBm* or *wPip* (B).

evolutionary past within their common invertebrate host, the cat flea *Ctenophalides felis* (Casiraghi *et al.*, 2005; Rolain *et al.*, 2003).

Ankyrin repeat proteins and confirmation of mCGH results

The sequencing and annotation of the *wMel* genome revealed 23 genes encoding ankyrin domain repeats, one of the highest levels in a prokaryotic genome (Wu *et al.*, 2004). The functions of these proteins in *wMel* are not known, although ankyrin repeats often promote protein–protein interactions. In eukaryotes, they can be involved in many functions, including cell cycle regulation and cytoskeleton interactions (Voronin & Kiseleva, 2007; Wu *et al.*, 2004), making them candidates for mediating *Wolbachia*–host interaction and host reproductive phenotypes (Foster *et al.*, 2005; Iturbe-Ormaetxe *et al.*, 2005a). In other symbiotic relationships, ankyrin repeat genes have been shown to bind to host chromatin in *A. phagocytophilum* (Catuogregli *et al.*, 2000; Park *et al.*, 2004), have a role in hydrogen peroxide resistance of *Pseudomonas aeruginosa* (Howell *et al.*, 2000) and be key in establishing a plant–bacterium relationship between *Lotus japonicus* and *Mesorhizobium loti* (Kumagai *et al.*, 2007).

The ankyrin protein content of nine *Wolbachia* strains was determined previously using hybridization and PCR amplification (Iturbe-Ormaetxe *et al.*, 2005a). A comparison of the two strains overlapping with this current study reveals a high degree of agreement. Using a ratio cutoff of 3 and with the exception of WD_0633, the results for strain *wAu* are in agreement with those previous results, revealing that all of the examined ankyrin genes from *wMel* are present in *wAu*. In strain *wRi*, three genes (WD_0566, WD_0147, WD_0754) were previously demonstrated as being present in *wRi* but are suggested to be absent/divergent in the current mCGH study. There are several possible explanations for these differences, including: (1) size and sequence of the hybridization probe/target pairs in the two studies, (2) sensitivity, particularly pertaining to single- versus dual-channel hybridization experiments, (3) cutoff criteria for assessing background, (4) data normalization, (5) hybridization stringency, or (6) polymorphic populations. However, despite these differences there is a high degree of concordance in the dataset.

In the current study, only *wRi* and *wSim* had an overrepresentation of absent/divergent ankyrin genes (Fisher's exact test, $P < 0.01$). Phage activity, along with the movement of transposable genetic elements, may help to explain the marked changes in the ankyrin gene content, as most absent/divergent ankyrin repeat genes were localized to the prophage WO-A in these two genomes. However, this does not reflect an absence of the prophage, as WO-A structural genes and some conserved hypothetical genes are conserved (Fig. 4B). In contrast, *wSan* contains the ankyrin repeat proteins in WO-A, but has missing/divergent phage components (Fig. 4B). Instead of prophage absence, this

likely reflects either differential loss of portions of the prophage region after incorporation into the genome and/or independent acquisition of the ankyrin genes. There appears to be a similar, but lower-level, variation in the WO-B ankyrin repeat genes in *wSan* and *wInn* (Fig. 4B), suggesting the presence of related prophage regions in *Wolbachia* strains with differing content of ankyrin repeat proteins.

Hypothetical proteins

Hypothetical proteins are also overrepresented in the absent/divergent genes in this study (Table 2). There are 72 hypothetical proteins ranging in size from 30 to 2843 aa with a ratio > 3 , suggesting absence/divergence in at least one strain. All of these proteins were re-examined in order to improve annotation. There are 19 (26%) that are conserved with *wPip*, but 66 (92%) remain hypothetical proteins unique to arthropod *Wolbachia*. Of the remaining six genes, three have orthologues in the *W. pipientis* *wBm* genome (WD_0424, WD_0434, WD_1039), one has an orthologue in the *Rickettsia felis* genome (WD_0268), one has an orthologue in *Ehrlichia chaffeensis* (WD_0971), and one has orthologues in *Anopheles gambiae* and *Aedes aegypti* (WD_0513). A third of the absent/divergent hypothetical proteins are > 150 aa, while the mean and median sizes are 209 aa and 68 aa, respectively. By comparison, the hypothetical proteins in the entire genome have the same size range but with mean and median sizes of 180 aa and 95 aa, respectively. Therefore, it is not merely the smallest hypothetical proteins, which one might hypothesize are incorrectly predicted to encode proteins, that are absent/divergent. As the unique genes in the genome, those encoding hypothetical proteins may reflect the genetic diversity of *Wolbachia*.

Divergence of strains

Most gene differences were unique to a strain, resulting in a unique set of absent/divergent genes per strain (Fig. 2A). Only three genes were present in *wMel* but absent/divergent in all other strains examined. These adjacent genes include a DNA mismatch repair protein MutL-2 (WD_0509), a degenerate RNase (WD_0510), and a conserved hypothetical protein (WD_0511). The region from WD_0509 to WD_0514 was previously hypothesized to be horizontally transferred into *wMel* based on absence in the *Wolbachia* draft genomes of *wAna* and *wSim* (Salzberg *et al.*, 2005). In the current study, WD_0512 and WD_0513 are also absent/divergent in *wAu* and showed higher than average ratios in all strains but *wSim*. Using PCR and Southern hybridization, WD_0509–WD_0514 were found to be absent in *wAu* and *wRi* while only partially absent in *wSan*, confirming the mCGH studies. In *wSim*, these WD_0512 and WD_0513 genes actually showed lower than average ratios, suggesting multiple copies. However, these regions are absent in the *D. simulans* genome sequencing project and corresponding

wSim *Wolbachia* draft genome (Salzberg *et al.*, 2005). These discrepancies may be attributed to a cloning bias in the whole-genome shotgun sequencing project or a *Wolbachia* population polymorphic for this region. Although unusual, a polymorphic population has some precedent. In the sequencing of *wPip*, the laboratory, inbred mosquito line used was not clonal, leading to the suggestion that *Wolbachia* can rapidly generate chromosomal variation (Klasson *et al.*, 2008).

WD_0513 is interesting as its closest putative homologues are found in the genomes of *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus* (Woolfit *et al.*, 2009) and facilitate invasion of malarial sporozoites into salivary gland cells (Arca *et al.*, 2005; Korochkina *et al.*, 2006). Prior analyses revealed that the co-transcribed WD_0512–WD_0514 genes (Iturbe-Ormaetxe *et al.*, 2005a) are frequently absent from *Wolbachia* genomes with the

exception of *wPip* (Korochkina *et al.*, 2006) and *Wolbachia* closely related to *wMel* including *wMelCS* and *wMelPop* (Iturbe-Ormaetxe *et al.*, 2005a). The limited distribution of these proteins in only mosquitoes and select *Wolbachia* strains suggests host/endosymbiont lateral gene transfer. Lateral transfer of *Wolbachia* genes to animal hosts has been described (Dunning Hotopp *et al.*, 2007; Kondo *et al.*, 2002), but in the case of WD_0513, the actual direction of this transfer is difficult to ascertain without further knowledge of the gene's phylogenetic distribution (Woolfit *et al.*, 2009). However, the limited distribution of this region and the presence of these proteins in both the endosymbiont and host make these genes interesting candidates for research into host–parasite interactions.

Although all of the strains examined here had distinct genomic content, *wRi* and *wSim* were found to be most similar (Figs 2A, 3 and 5). It has been hypothesized that

Fig. 5. Circular representation of mCGH results. The outer two rims present the predicted coding regions on the plus and minus strand, respectively, colour-coded by role categories. Rim 3 contains the genes not represented on the array (orange) and atypical nucleotide content (black). Rim 4 indicates the origin of replication (gold), phage genes (pink), and transposon genes (green). Rims 5–9 are the comparative genome hybridization results for *wAu*, *wInn*, *wRi*, *wSim* and *wSan*, respectively. Using previously determined cutoffs, the following assignments were made: a ratio >3 is most likely absent or divergent in the test strain (red); a ratio between 2 and 3 is absence/divergence not predicted (blue); a ratio <2 is most likely present in the test strain (white). Considerable diversity is present in the absent/divergent genes, particularly in the vicinity of phage (rim 4, pink).

these are actually the same strain (Iturbe-Ormaetxe *et al.*, 2005b), and the two strains are identical in their MLST alleles (Fig. 3). In this current study, the divergence of strains could not be determined using microarray support trees analysis as all nodes had <50 % support (TMeV support trees, average linkage, Euclidean distance, bootstrap 100 iterations; data not shown). However, these strains had the highest number of absent/divergent genes in common in all of the two-way comparisons (Fig. 2A). Despite this commonality, there are 46 differences in absent/divergent genes between the two strains while only 21 absent/divergent genes overlapped in these two strains (Fig. 2A), suggesting distinct genomic content.

DISCUSSION

Genome diversity

Although *Wolbachia* are obligate intracellular bacteria, they are considered facultative endosymbionts because hosts can survive uninfected. They are cosmopolitan, with host promiscuity and frequent co-infection with other *Wolbachia* strains and probably other intracellular bacteria. These factors combined likely increase the opportunity of *Wolbachia* to exchange genomic DNA. This increased genomic exchange may promote genetic and phenotypic diversity and contribute to *Wolbachia*'s success as one of the most abundant intracellular bacteria.

Lateral gene transfer

In the supergroup A and B *Wolbachia*, intraspecies lateral gene transfer is facilitated by prevalent infections (Hilgenboecker *et al.*, 2008), transfer of mobile elements (Bordenstein & Wernegreen, 2004; Masui *et al.*, 2000) and extensive recombination (Baldo *et al.*, 2006a, b; Lo *et al.*, 2007; Verne *et al.*, 2007). It has not been clear if *Wolbachia* can exchange DNA through lateral gene transfer with other bacteria. In order for this to happen, *Wolbachia* must come into contact with other bacteria either inside the cell or as it is passing between cells/organisms (e.g. in the haemolymph). We show here a region shared at ~70 % nucleotide identity flanked by transposases on a plasmid in a *Rickettsia* endosymbiont and flanked by prophage sequences in a *Wolbachia*. The direction of the transfer is difficult to identify. This region is not found in all *Rickettsia* spp. or all *Wolbachia* strains, and has no close similarity to other organisms to allow a phylogenetic assignment. Both organisms can infect ovaries and can infect *I. scapularis* (Benson *et al.*, 2004), possibly providing the opportunity for plasmid transfer. Alternatively, another host or a third DNA reservoir may be involved in mediating this transfer.

Limitations/benefits of mCGH

mCGH is of limited use in genomic comparison of intracellular bacteria with low genomic diversity. For

example, a comparison of two *A. phagocytophilum* genomes revealed that only four *p44* genes varied between the strains (Dunning Hotopp *et al.*, 2006b). These encompass a subset of genes that were already known to be highly variable. But in the case of *Wolbachia*, where large genomic variation is seen, mCGH is more fruitful.

The mCGH platform may become obsolete as sequencing costs fall, but with in-house printed arrays the cost is still an order of magnitude less than most commercial microarray platforms and two orders of magnitude less than data obtained through next-generation sequencing. So mCGH may still have significant use in assessing genomic diversity, particularly before selecting strains to be sequenced.

One problem with examining obligate intracellular bacteria with any genomic platform is acquiring microgram quantities of DNA that are devoid of host genomic DNA contamination. Better methods are needed to obtain purified material and to amplify materials when genomic DNA quantity is limited. In this current study, several strains had to be eliminated due to high levels of background hybridization, likely owing to host DNA contamination. Likewise, because of limiting quantities of highly purified *Wolbachia* DNA, whole-genome amplification could not be avoided. These limiting DNA quantities precluded assessment of bias in the current study, but the amplification results were shown to be reproducible. By pooling multiple amplification reactions, performing multiple replicate hybridizations, and taking the median value of those replicates any biases should be minimized.

One of the limitations of mCGH, when compared to sequencing, is that it only assesses gene content represented on the microarray. As such, only sequences represented in public databases are queried. Although great genomic diversity of *wMel* prophage regions is demonstrated, novel phage regions and genes cannot be assessed using the mCGH technique. The high levels of variation in these regions suggest that further sequencing of *Wolbachia* and its phage would be particularly fruitful.

Concluding remarks

In *Wolbachia*, mCGH can identify variable regions including phage regions, ankyrin repeat proteins, and hypothetical proteins. However, one of the limitations is the ability to only characterize the diversity relative to known gene content. The unique genes in each strain examined remain elusive. But even with this limitation, the difference in gene content is the greatest variation observed for closely related obligate intracellular bacteria, with *Wolbachia* phages being a major source of variation. *Wolbachia* sequencing has been stymied due to the high levels of host DNA contamination. But as the costs of genome sequencing decrease and new sequencing technologies are developed that alleviate this burden, *Wolbachia* strains should be targeted for further sequencing

to better understand their uniquely high levels of genetic diversity among endosymbionts.

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