Intense Transpositional Activity of Insertion Sequences in an Ancient Obligate Endosymbiont

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The streamlined genomes of ancient obligate endosymbionts generally lack transposable elements, such as insertion sequences (IS). Yet, the genome of Wolbachia, one of the most abundant bacterial endosymbionts on Earth, is littered with IS. Such a paradox raises the question as to why there are so many ISs in the genome of this ancient endosymbiont. To address this question, we investigated IS transpositional activity in the unculturale Wolbachia by tracking the evolutionary dynamics and history of ISWpi1 elements. We show that 1) ISWpi1 is widespread in Wolbachia, being present in at least 55% of the 40 sampled strains, 2) ISWpi1 copies exhibit virtually identical nucleotide sequences both within and among Wolbachia genomes and possess an intact transposase gene, 3) individual ISWpi1 copies are differentially inserted among Wolbachia genomes, and 4) ISWpi1 occurs at variable copy numbers among Wolbachia genomes. Collectively, our results provide compelling evidence for intense ISWpi1 transpositional activity and frequent ISWpi1 horizontal transmission among strains during recent Wolbachia evolution. Thus, the genomes of ancient obligate endosymbionts can carry high loads of functional and transpositionally active transposable elements. Our results also indicate that Wolbachia genomes have experienced multiple and temporally distinct ISWpi1 invasions during their evolutionary history. Such recurrent exposition to new IS invasions may explain, at least partly, the unusually high density of transposable elements found in the genomes of Wolbachia endosymbionts.

Introduction

Insertion sequences (ISs) are prokaryotic autonomous transposable elements that encode a transposase gene mediating their transposition (i.e., their ability to move to another locus in a genome) (Chandler and Mahillon 2002). ISs are widespread among prokaryotic genomes (e.g., present in >75% of 262 representative genomes surveyed; Touchon and Rocha 2007), in which they can represent substantial proportions (Chandler and Mahillon 2002; Siguier et al. 2006; Filee et al. 2007). However, when host lifestyle is considered, it is notable that ISs are largely missing from the genomes of obligate endosymbionts, which is, intracellular bacteria that replicate exclusively in the cells of other organisms and typically have no extracellular state (Moran and Plague 2004; Bordenstein and Reznikoff 2005; Touchon and Rocha 2007). This is generally ascribed to the confined and isolated intracellular environment in which these bacteria reside, which reduces opportunities for acquisition of genetic material. This view is supported by the strikingly stable genomes of various obligate endosymbionts of insects such as Buchnera, which lack IS and have experienced no genomic rearrangement and gene acquisition for the past 50–70 Myr (Tamas et al. 2002). Yet, comparative genomic analyses of various Rickettsiales, a diverse group of intracellular alpha-Proteobacteria, have demonstrated striking exceptions to this pattern in which these genomes exhibit extensive variability in their mobile element content, including IS (Darby et al. 2007). However, the within-species IS dynamics has not been studied for this group of bacteria, making difficult the analysis of the microevolutionary events responsible for this variability.

Within Rickettsiales, Wolbachia bacteria are ancient obligate endosymbionts that have been associated with arthropod and nematode hosts for >100 Myr (Rousset et al. 1992; O’Neill et al. 1997; Bandi et al. 1998; Bourtzis and Miller 2003) and possibly represent one of the most abundant bacterial endosymbionts on Earth (Werren, Windsor, and Guo 1995). These maternally inherited bacteria are often referred to as reproductive parasites because they are able to manipulate the reproduction of their arthropod hosts to increase their own transmission (O’Neill et al. 1997; Bourtzis and Miller 2003; Cordaux et al. 2004). In addition to vertical transmission, Wolbachia from arthropods are occasionally transmitted horizontally (Werren, Zhang, and Guo 1995; Vavre et al. 1999; Cordaux et al. 2001). Contrary to expectations, genome sequencing of the Wolbachia strain harbored by the fruit fly Drosophila melanogaster (wMel) revealed an unusually high proportion of repetitive and mobile DNA, including IS (Moran and Plague 2004; Wu et al. 2004; Bordenstein and Reznikoff 2005). This result is particularly significant given that wMel otherwise exhibits many typical features of a long-term symbiotic lifestyle, such as reduced genome size and A + T nucleotide composition richness (Wernegreen 2002; Wu et al. 2004). Such a paradox raises the question as to why there are so many ISs in the genome of this endosymbiont.

To address this question, we investigated IS transpositional activity in the unculturale Wolbachia by tracking the evolutionary dynamics and history of ISWpi1, a group of IS related to the IS5 family, the distribution of which is so far exclusively restricted to Wolbachia bacteria (Cordaux 2008). Previous results suggest that ISWpi1 transposase may potentially be functional because 1) the 2 overlapping open reading frames constituting ISWpi1 transpose are intact in many copies (Cordaux 2008) and 2) several ISWpi1 copies are differentially inserted in various Wolbachia strains (Duron et al. 2005; Iturbe-Ormaetxe et al. 2005; Riegler et al. 2005). Here, we show that Wolbachia endosymbionts have recently experienced, and probably continue to experience, high levels of ISWpi1 transpositional activity.
within genomes and horizontal transfers among genomes. Our results thus provide compelling evidence that ancient obligate endosymbionts can carry high loads of functional and transpositionally active transposable elements. This may explain, at least partly, why the genomes of Wolbachia endosymbionts are littered with IS.

Materials and Methods

Wolbachia Strains

Forty Wolbachia strains identified from 23 insect (5 different orders), 13 crustacean (3 different orders), and 4 arachnid individual hosts were used (table 1). Some animals originated from laboratory strains, whereas others were caught in the wild. Total DNA was extracted as previously described (Bouchon et al. 1998). To confirm the presence of Wolbachia DNA of suitable quality in the samples, 2–3 loci from Wolbachia chromosomal DNA (wsp, 16S rRNA, and GroE) were amplified by polymerase chain reaction (PCR), as previously described (Bouchon et al. 1998; Cordaux et al. 2001; Verne et al. 2007). Purified wsp PCR products were directly sequenced as previously described (Cordaux et al. 2001). Each of the 40 samples was infected by a single Wolbachia strain, as indicated by the lack of ambiguity in the electrophoregrams. Sequences generated in this study were deposited in GenBank under accession numbers EU288004–EU288015.

Table 1

| Host Species (Strain) | Taxonomic Group | Geographic Origin | Wolbachia Supergroup | ISWpi1 Presence |
|-----------------------|-----------------|------------------|----------------------|-----------------|
| Alesochara bilineata  | Insecta, Coleoptera | Canada           | A                    | Yes             |
| Delta radicum        | Insecta, Diptera | Brittany, France | A                    | Yes             |
| Drosophila ananassae | Insecta, Diptera | Rio de Janeiro, Brazil | A                  | Yes             |
| Drosophila auraria   | Insecta, Diptera | Tokyo, Japan     | A                    | Yes             |
| Drosophila melanogaster (wMel)* | Insecta, Diptera | Antibes, France | A                    | Yes             |
| Drosophila simulans (wAu) | Insecta, Diptera | Yaounde, Cameroon | A                        | Yes             |
| D. simulans (wRi)    | Insecta, Diptera | Antibes, France  | A                    | Yes             |
| Drosophila suzukii   | Insecta, Diptera | Tokyo, Japan     | A                    | Yes             |
| Drosophila triauraria| Insecta, Diptera | Tokyo, Japan     | A                    | Yes             |
| Drosophila yakuba    | Insecta, Diptera | Ougoue River, Gabon | A                   | Yes             |
| Zaprionus seposoides | Insecta, Diptera | Sao Tomé         | A                    | Yes             |
| Asobara tabida (wAtab3) | Insecta, Hymenoptera | Antibes, France | A                    | Yes             |
| Asobara japonica     | Insecta, Hymenoptera | Sapporo, Japan   | A                    | Yes             |
| Lepopillina heterotoma (wLhet1) | Insecta, Hymenoptera | Antibes, France | A                    | Yes             |
| Pachycrepoideus dubs | Insecta, Hymenoptera | France         | A                    | Yes             |
| Amaurobius ferox     | Arachnida, Araneae | Poitiers, France | B                    | Yes             |
| Segestria florentina | Arachnida, Araneae | Chizé, France    | B                    | No              |
| Talitrus saltator    | Crustacea, Amphipoda | La Rochelle, France | B                        | Yes             |
| Lepas anatigera      | Crustacea, Cirripedia | La Rochelle, France | B                        | No              |
| Armadillidium vulgare (wVuI) | Crustacea, Isopoda | Saint Cyr, France | B                    | Relic only      |
| A. vulgare (wVuM)    | Crustacea, Isopoda | Méry sur Cher, France | B                      | Relic only      |
| Cylisticus convexus  | Crustacea, Isopoda | Avanton, France  | B                    | Relic only      |
| Heterilia brevicornis| Crustacea, Isopoda | Bastia, France   | B                    | No              |
| Oniscus asellus      | Crustacea, Isopoda | Golbey, France   | B                    | Relic only      |
| Philoscia muscorum   | Crustacea, Isopoda | Poitiers, France | B                    | No              |
| Platysarthrus hoffmannseggii | Crustacea, Isopoda | Liniers, France | B                    | Yes             |
| Porcellio dilatatus petitis | Crustacea, Isopoda | Saint Honorat, France | B                        | No              |
| Porcellionides pruinosus (wPruIII) | Crustacea, Isopoda | Nevers, France | B                    | Relic only      |
| Sphaeroma hookeri    | Crustacea, Isopoda | Graye sur Mer, France | B                    | No              |
| Sphaeroma rugicauca  | Crustacea, Isopoda | Alresford Creek, United Kingdom | B                      | No              |
| Drosophila sechellia (wSn) | Insecta, Diptera | Seychelles Archipelago | B                      | Yes             |
| Reticulitermes santonensis | Insecta, Isoptera | Charante, France | B                    | Yes             |
| Charanyma trigrammica | Insecta, Lepidoptera | Pineau, France | B                    | No              |
| Lomaspilis marginata | Insecta, Lepidoptera | Poitiers, France | B                    | No              |
| Maniola jurtina      | Insecta, Lepidoptera | Poitiers, France | B                    | No              |
| Peribatodes rhomboidaria | Insecta, Lepidoptera | Poitiers, France | B                    | Yes             |
| Spilosoma lubricipeda | Insecta, Lepidoptera | Poitiers, France | B                    | No              |
| Dysdera crocuta      | Arachnida, Araneae | Chizé, France    | G                    | No              |
| Dysdera erythrina    | Arachnida, Araneae | Saint Benoit, France | G                     | No              |
| Musca domestica      | Insecta, Diptera | Poitiers, France | G                    | No              |
| Water control        | —                | —                | —                    | No              |

* Used as a positive control because ISWpi1 presence is confirmed by in silico analyses (Wu et al. 2004; Cordaux 2008).

ISWpi1 Detection Assay

To investigate the distribution of ISWpi1 among the 40 Wolbachia strains, we designed an intra-ISWpi1 PCR assay, using primers internal to the ISWpi1 consensus sequence. A 681-bp long region internal to ISWpi1 was amplified using specific oligonucleotide primers ISWpi1-F (5'–GATCTAAGCGAAAGGGAATGG) and ISWpi1-R (5’–CAACCCATCTTCTTTGGCTTGTG), PCR amplification was performed using a standard protocol, with an annealing temperature of 60 °C (Cordaux et al. 2006). Resulting PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized using UV fluorescence. To confirm the results, PCR amplifications were performed...
at least twice for each sample, and purified PCR products were directly sequenced as above. ISWpi1 sequences were deposited in GenBank under accession numbers EU288016–EU288038 and EU684314–EU684317. To further confirm the results, Wolbachia strains inferred to lack ISWpi1 based on the above PCR assay were subjected to a second PCR assay amplifying 197 bp of ISWpi1 internal sequence, using specific oligonucleotide primers ISWpi1-F1 (5'-CGAAAGGGATGGTCACAGA and ISWpi1-R1 (5'-GCTCTTTCCTATGCCTGAAC) and an annealing temperature of 54 °C.

ISWpi1 Locus Genotyping

To evaluate the timing of ISWpi1 transpositional activity during Wolbachia evolution, we assessed the "presence" or "absence" of 24 ISWpi1 copies at orthologous genomic sites in 16 A-supergroup Wolbachia strains. Nucleotide sequences of 24 different ISWpi1 copies identified from the wMel, wAna, wSim, and wWil Wolbachia genomes (Wu et al. 2004; Salzberg, Dunning Hotopp, et al. 2005; Salzberg, Hotopp, et al. 2005; Cordaux 2008) were downloaded from GenBank along with 500 bp of genomic sequence flanking each element on both sides (when available). Specific oligonucleotide primers were designed in the flanking sequences of each ISWpi1 copy, according to the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The presence or absence of the 24 ISWpi1 copies was investigated in 12 A-supergroup Wolbachia strains from table 1 (strains from Delia radicum, Drosophila suzukii, and Pachycycrepoideus dubius were excluded because of insufficient amounts of DNA) using locus-specific PCR assays and confirmed by sequencing of the resulting PCR products, as described above. PCR conditions for each locus, including primer sequences and expected PCR product sizes, are shown in supplementary table S1 (Supplementary Material online). Two loci (wMel#4 and wMel#9 in supplementary table S1, Supplementary Material online) had to be discarded for further analyses because PCR amplification was successful only in the wMel sample. No case of double amplification of expected PCR products for both presence and absence alleles was observed, suggesting homogeneity of the Wolbachia population within individual hosts. Sequences were deposited in GenBank under accession numbers EU714507–EU714683. In addition, we performed in silico PCR for 4 A-supergroup Wolbachia strains for which genome sequence is available: wMel, wAna, wSim, and wWil (Wu et al. 2004; Salzberg, Dunning Hotopp, et al. 2005; Salzberg, Hotopp, et al. 2005; Cordaux 2008).

Southern Blotting

To assess ISWpi1 copy number variation among Wolbachia strains, approximately 5 µg of total DNA from various samples were digested with HindIII at 37 °C overnight. HindIII was chosen because in silico digestion of the ISWpi1 genome predicted the 13 wMel ISWpi1 copies to be located on different digested genomic fragments of relevant sizes. Digested DNA was size fractionated on 1% agarose gels and Southern blotted to nylon membranes. Probes were prepared as internal portions of ISWpi1 amplified by PCR using the aforementioned primers ISWpi1-F and ISWpi1-R. PCR products were labeled using [α-32P]-deoxyctydine triphosphate by the random primer method and hybridized overnight to membranes. The final wash was at 52 °C in 0.1 × standard saline citrate. Hybridized blots were imaged and analyzed using a Phospholmager (Molecular Dynamics, Sunnyvale, CA).

Sequence Analyses

Sequences were aligned using ClustaW as implemented in the software Bioedits version 7.0 (Hall 1999), followed by manual adjustments. MEGA version 4 (Tamura et al. 2007) was used to calculate nucleotide sequence divergence and build A- and B-supergroup Wolbachia phylogenetic trees using distance-based (Neighbor-Joining [NJ] and unweighted pair group method with arithmetic mean) and character-based (maximum parsimony [MP]) methods. The different methods yielded largely congruent phylogenies, and we showed in the paper the trees that displayed the highest confidence levels in branching patterns, as detailed below.

Due to low genetic differentiation among strains (Warren, Zhang, and Guo 1995), distance-based methods yielded A-supergroup Wolbachia trees with mostly short branches and low confidence in the branching patterns (i.e., low bootstrap scores). By contrast, MP yielded only 5 equally most parsimonious trees (tree length: 875 steps) that differed only in the branching patterns of the 4 highly closely related Wolbachia strains from Drosophila simulans (wRi and wSim variants) and Drosophila ananassae (2 wAna variants). Overall, this suggested high support for the branching patterns of the MP inference. Based on prior knowledge on strain origins, the most parsimonious tree linking the 2 D. simulans Wolbachia variants, on the one hand, and the 2 D. ananassae Wolbachia variants, on the other hand, was considered as most biologically relevant tree. The high consistency index (0.875) provided further support for the MP tree shown in figure 1.

Regarding B-supergroup Wolbachia strains, distance-based and MP trees essentially differed on the position of the Reticulitermes santonensis Wolbachia strain. However, the MP analysis yielded as many as 190 equally parsimonious trees (tree length: 440 steps), with a consistency index of only 0.745. By contrast, the 2 distance-based methods (which agreed on the branching pattern of the R. santonensis Wolbachia strain) were characterized by high bootstrap scores. Hence, 10 out of 14 nodes displayed bootstrap values >95% in the NJ tree, thus providing strong support for the NJ topology.

Results and Discussion

Widespread Distribution of ISWpi1 among Wolbachia Strains

The taxonomic distribution of ISWpi1 is apparently restricted to Wolbachia bacteria, as found earlier by Blast
searches against the entire GenBank database and all prokaryote genomes listed in the ‘microbial genomes’ section of GenBank (Cordaux 2008). In this study, we confirm ISWpi1 restricted distribution even though new sequence data have been added to GenBank since previous searches. Using a PCR-based ISWpi1 detection assay, we screened a panel of 40 diverse Wolbachia strains belonging to the A, B, and G Wolbachia supergroups (table 1). A PCR fragment of the expected size (681 bp) was obtained in 22 out of the 40 tested Wolbachia strains. Absence of the expected 681-bp long PCR fragment in some strains is unlikely to be caused by systematic PCR failure due to primer mismatches because average ISWpi1 sequence divergence across 22 Wolbachia strains is only 0.22% (see below), indicating that 2 full-length ISWpi1 sequences are expected to differ by only 2 substitutions on average. Moreover, Wolbachia strains inferred to lack ISWpi1 based on the first PCR assay were subjected to a second ISWpi1 detection assay, which confirmed the initial results.

ISWpi1 was not uniformly distributed among Wolbachia supergroups (P < 10^-5, Fisher’s exact test). It was present in all 15 A-supergroup Wolbachia strains screened (table 1), in agreement with its presence in all A-supergroup Wolbachia strains for which genomic information is available (Cordaux 2008). By contrast, ISWpi1 was found in only 32% (7/22) of B-supergroup and none (0/3) of the G-supergroup Wolbachia strains tested (table 1). Overall, these results indicate that ISWpi1 is widespread among Wolbachia endosymbionts because it is present in the genomes of 55% of all Wolbachia strains tested.

Extreme ISWpi1 Sequence Homogeneity within and among Wolbachia Strains

To investigate ISWpi1 nucleotide variation, we compared the ISWpi1 sequences obtained from the 22 Wolbachia strains identified above as possessing ISWpi1. PCR products were directly sequenced to simultaneously sequence homologous regions from multiple ISWpi1 copies possibly occurring within a single Wolbachia genome. Lack of ambiguous sites in the sequence trace files suggested extremely low to no nucleotide variation within a single Wolbachia genome. Lack of ambiguous sites in the sequence trace files suggested extremely low to no nucleotide divergence among the different ISWpi1 copies occurring within each Wolbachia genome. This result is consistent with the virtual lack of nucleotide variation previously recorded among the ISWpi1 copies present within various sequenced Wolbachia genomes (Cordaux 2008). However, some private substitutions might have remained undetected with this sequencing strategy. Thus, the 22 ISWpi1 sequences can actually be viewed as consensus sequences of all individual ISWpi1 copies inserted within each of the analyzed Wolbachia genomes, making them useful for comparisons among strains. Overall, nucleotide divergence of the 22 ISWpi1 sequences from the various A- and B-supergroup Wolbachia strains was only 0.22%. This virtual lack of ISWpi1 sequence variation among Wolbachia genomes is in sharp

Fig. 1.—Distribution of 22 ISWpi1 copies isolated from the wMel (red), wWil (green), wSim (brown), and wAna (blue) reference genome sequences. Colored circles highlight the numbers of inferred absence/presence transitions of ISWpi1 copies in different branches of the phylogenetic tree of 16 A-supergroup Wolbachia strains. The tree was reconstructed by MP (based on 9,782 bp of sequence flanking the 22 ISWpi1 loci and the wsp gene) and rooted using the B-supergroup Wolbachia strain from Culex pipiens (Sanger Institute, http://www.sanger.ac.uk/Projects/W_pipientis). Branch length is arbitrary. Wolbachia strains are identified by the host species from which they were isolated.
contrast with the ~3.7% average nucleotide divergence among \textit{Wolbachia} supergroups A and B recorded for 8 highly conserved housekeeping genes (range: 2.2–4.9%) and even much lower than the divergence (~0.7%) observed for the extremely conserved 16S rRNA gene (Paraskevopoulos et al. 2006).

Purifying selection acting on ISWpi1 transposase genes is unlikely to account for this extreme ISWpi1 sequence homogeneity because it would imply that selection for transposition is stronger than selection constraining housekeeping genes essential for \textit{Wolbachia} metabolism. Maintaining such intense levels of purifying selection on ISWpi1 sequences seems further implausible given the elevated evolutionary rates and relative inefficiency of natural selection in endosymbiotic bacteria with reduced effective population sizes, such as \textit{Wolbachia} (Wu et al. 2004). Gene conversion (i.e., the nonindependent evolution of repetitive DNA sequences) could explain the homogeneity of ISWpi1 copies within \textit{Wolbachia} genomes, but it cannot account for the homogeneity of ISWpi1 among \textit{Wolbachia} genomes. Therefore, the most likely explanation for the presence of highly homogeneous ISWpi1 sequences in \textit{Wolbachia} strains as divergent as those belonging to different supergroups is that ISWpi1 has been transpositionally active and laterally acquired by diverse \textit{Wolbachia} strains during very recent evolutionary times (Wagner 2006).

Recent and Intense ISWpi1 Transpositional Activity

To evaluate the timing of ISWpi1 transpositional activity during \textit{Wolbachia} evolution, we analyzed the phylogenetic distribution of 22 individual ISWpi1 copies in 16 A-supergroup \textit{Wolbachia} strains. This approach allowed us to pinpoint transitions between absence and presence of individual ISWpi1 copies, which are signatures of transpositional activity, during A-supergroup \textit{Wolbachia} evolutionary history. Some transitions might have been overlooked because ISWpi1 status could not be determined for some loci in some taxa. We emphasize, however, that it would not affect our conclusions based on a conservative set of unambiguously determined transitions.

We were able to map presence/absence transitions to the \textit{Wolbachia} phylogeny for 11 wMel ISWpi1 copies. Our results indicated that none of the ISWpi1 copies is shared by all A-supergroup \textit{Wolbachia} strains (fig. 1 and supplementary table S2 [Supplementary Material online]). Instead, all copies showed very narrow strain distributions. Hence, 7 ISWpi1 copies identified from the wMel genome sequence were apparently specific to wMel. The other copies were shared with just a few closely related \textit{Wolbachia} strains that exhibit >99% nucleotide sequence identity with wMel based on the hypervariable \textit{Wolbachia}-specific \textit{wsp} gene (Charlat et al. 2003, 2004). In fact, 2 copies have presumably been transpositionally active so recently in wMel that they are polymorphic for insertion presence or absence among different geographic wMel variants. Specifically, ISWpi1 copies at loci wMel#6 and wMel#12 isolated from the sequenced wMel genome (Wu et al. 2004; Cordaux 2008) were absent from our wMel sample originating from France. Although wMel#6 (WD0516-0517 in the original wMel genome annotation) has previously been shown to be polymorphic (Riegler et al. 2005), we identified here wMel#12 as a novel polymorphic marker that may prove useful for studies of \textit{Wolbachia} diversity and evolution in \textit{D. melanogaster}.

To test if the very recent ISWpi1 transpositional activity suggested by the transition patterns of ISWpi1 copies isolated from wMel can be generalized to other ISWpi1 copies, we extended our analysis to 11 additional ISWpi1 copies isolated from the partial genome sequences of wAna (6 loci), wSim (2 loci), and wWil (3 loci). Again, all ISWpi1 copies exhibited very narrow strain distributions (fig. 1 and supplementary table S2 [Supplementary Material online]). Even the 2 most widely distributed ISWpi1 copies isolated from wAna were found in closely related \textit{Wolbachia} strains that are identical based on the hypervariable \textit{Wolbachia}-specific \textit{wsp} gene (Miller and Riegler 2006).

Next, we assessed ISWpi1 copy number variation among A-supergroup \textit{Wolbachia} strains by Southern blotting. Results indicated that the number of distinct bands (i.e., putative distinct copies) for A-supergroup \textit{Wolbachia} strains varies from 7 to 13 copies (fig. 2). These figures are in line with the copy numbers estimated from genome sequence data for other A-supergroup \textit{Wolbachia} strains (Cordaux 2008). Interestingly, there are approximately twice as many ISWpi1 copies in wMel compared with the closely related wAu, whereas there are similar copy numbers between wMel and the distantly related wAna (fig. 2 and Cordaux 2008).

Overall, extensive heterogeneity in ISWpi1 copy numbers among \textit{Wolbachia} strains, along with very narrow distribution of 22 individual ISWpi1 copies identified from 4 different host genomes and extreme ISWpi1 sequence homogeneity, provides compelling evidence for intense ISWpi1 transpositional activity during recent \textit{Wolbachia} evolution. We emphasize that the extensive polymorphism observed, both in terms of overall copy numbers and patterns of presence or absence of individual copies among \textit{Wolbachia} strains, may result from a combination of insertion events and secondary excisions. In any event, this testifies to the intense transpositional activity that \textit{Wolbachia} endosymbionts have recently experienced and may continue to currently experience. ISWpi1 recent transposition in various \textit{Wolbachia} strains is further supported by the fact that the 2 overlapping open reading frames constituting ISWpi1 transposases are intact in all sequenced portions, suggesting that there are sources of functional transposases in all A- and B-supergroup \textit{Wolbachia} genomes containing ISWpi1 we analyzed. If so, our results provide strong evidence that the genomes of ancient obligate endosymbionts can carry high loads of functional and active transposable elements.

Frequent ISWpi1 Horizontal Transfers during Recent \textit{Wolbachia} Evolution

The ubiquitous presence of ISWpi1 in the \textit{Wolbachia} A supergroup, coupled with reduced levels of sharing of individual copies among \textit{Wolbachia} strains, suggests that some \textit{Wolbachia} strains may have independently acquired
ISWpi1 via lateral transfers. To estimate the number of independent ISWpi1 acquisitions in the Wolbachia B supergroup, we analyzed ISWpi1 distribution according to bacterial strain phylogenetic relationships (fig. 3). At this level of resolution, the presence of ISWpi1 in B-supergroup Wolbachia strains putatively results from at least 4 independent acquisitions (fig. 3). This may be an underestimate because 1) a higher phylogenetic resolution in the Lomaspilis marginata/Talitrus saltator/Amaurobius ferox group of closely related Wolbachia strains might result in the inference of additional independent ISWpi1 acquisitions, 2) a larger screening of Wolbachia strains for ISWpi1 presence might uncover additional acquisition events, and 3) one cannot formally exclude that ISWpi1 has been transferred several times to individual Wolbachia strains. In any event, these results suggest that horizontal transmission may be a major determinant of the current ISWpi1 distribution among Wolbachia strains. Only limited cases of horizontal transfers of mobile DNA in obligate endosymbiotic bacteria have been reported previously, including a plasmid in Buchnera (Van Ham et al. 2000), a bacteriophage in Wolbachia (Bordenstein and Wernegreen 2004; Gavotte et al. 2007), and a putative conjugative element in Rickettsia (Blanc et al. 2007). ISWpi1 from Wolbachia is the first transposable element unambiguously shown to horizontally transfer in obligate endosymbiotic bacteria.

Frequent ISWpi1 transfers among different Wolbachia strains could be facilitated by the occasional co-occurrence of divergent Wolbachia strains within the same host cells (Vavre et al. 1999; Bordenstein and Wernegreen 2004), as well as the presence of bacteriophage WO in many Wolbachia genomes (Bordenstein and Wernegreen 2004; Wu et al. 2004; Braquart-Varnier et al. 2005; Gavotte et al. 2007) that could serve as a shuttle for efficiently transferring genetic material among strains. Consistently, the wBm Wolbachia genome from the nematode Brugia malayi that lacks bacteriophage WO (Foster et al. 2005) also lacks recent ISWpi1 copies (Cordaux 2008). On the other hand, bacteriophage WO distribution seems restricted to Wolbachia, and it has never been found in other bacteria to date (Bordenstein and Wernegreen 2004; Gavotte et al. 2007), which could also contribute to explain why ISWpi1 taxonomic distribution also appears restricted to Wolbachia (Cordaux 2008). If so, Wolbachia bacteria may constitute a highly dynamic system for genetic exchanges among strains (Bordenstein and Wernegreen 2004), whereas at the same time being less prone to exchanges with other bacterial species, perhaps as a result of the specialization of vectors involved in IS horizontal transfer.

Why So Many ISs in Wolbachia Genomes?

While investigating ISWpi1 distribution by PCR in 40 Wolbachia strains, we amplified ISWpi1 “relics” from the genomes of 5 B-supergroup Wolbachia strains: a 312-bp fragment in 4 Wolbachia strains (including wVulC) and a 550-bp fragment in 1 Wolbachia strain (table 1). DNA sequencing revealed that the shorter and longer fragments exhibited 12.3% and 10.4% nucleotide divergence with ISWpi1, respectively, and 20.1% with each other. In addition, both fragments were severely truncated compared with ISWpi1 due to multiple internal deletions and both were lacking any significant coding capacity. Southern blotting of wVulC Wolbachia strain DNA against an ISWpi1 probe identified a single band (fig. 2), suggesting that the ISWpi1 relic identified above is the only ISWpi1 copy currently inserted in the wVulC genome. Other highly divergent copies have also been reported from the B-supergroup wPip and D-supergroup wBm Wolbachia strains (Duron et al. 2005; Cordaux 2008), suggesting an ancient presence of ISWpi1 in Wolbachia genomes. Because our PCR-based strategy was designed to preferentially detect ISWpi1 copies closely related to the ISWpi1 consensus sequence (i.e., presumably recent copies), it is possible that some ISWpi1 relics have remained undetected in our screening. Thus, the distribution of ISWpi1 relics among Wolbachia genomes may be underestimated.
Overall, our results are consistent with a scenario in which ISs recurrently invade and then go extinct in bacterial genomes (Wagner 2006) so that ancient relics and recent ISWpi1 copies represent temporally distinct ISWpi1 invasions of Wolbachia genomes. It has been proposed that IS could be maintained in Wolbachia genomes because they confer a selective advantage to their bacterial hosts (Brownlie and O'Neill 2005; Foster et al. 2005). Alternatively, it is possible that ISs are maintained in Wolbachia simply as a consequence of the inefficiency of host genomes to eliminate them (Wu et al. 2004). The rationale underlying this hypothesis is that symbiotic bacteria tend to have small effective population sizes, thus rendering selection against deleterious mutations and transposable element insertions less efficient (Wu et al. 2004). The evolutionary history and dynamics of ISWpi1 suggest yet another explanation: Wolbachia genomes are recurrently exposed to new IS invasions (Bordenstein and Werneckgreen 2004).

Conclusion

It is generally considered that IS proliferation characterizes lineages that have recently evolved toward an obligate endosymbiotic lifestyle (Moran and Plague 2004; Plague et al. 2008). By contrast, ancient obligate endosymbionts typically lack IS because of degradation of old insertions and absence of exposure to new transposition events (Moran and Plague 2004). Unexpectedly, our results show that at least a subset of all IS copies of the obligate endosymbiont Wolbachia are not remnants of ancient IS proliferation following the shift to endosymbiotic lifestyle at an earlier stage of Wolbachia evolution. Instead, Wolbachia experienced recurrent invasions by new IS, which may explain, at least partly, the unusually high density of transposable elements found in the genomes of these endosymbionts.

Supplementary Material

Supplementary tables S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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