Walker, T; Klasson, L; Sebaihia, M; Sanders, MJ; Thomson, NR; Parkhill, J; Sinkins, SP (2007) Ankyrin repeat domain-encoding genes in the wPip strain of Wolbachia from the Culex pipiens group. BMC biology, 5. p. 39. ISSN 1741-7007 DOI: https://doi.org/10.1186/1741-7007-5-39

Downloaded from: http://researchonline.lshtm.ac.uk/1620558/

DOI: 10.1186/1741-7007-5-39

Usage Guidelines

Please refer to usage guidelines at http://researchonline.lshtm.ac.uk/policies.html or alternatively contact researchonline@lshtm.ac.uk.

Available under license: http://creativecommons.org/licenses/by/2.5/
Ankyrin repeat domain-encoding genes in the wPip strain of Wolbachia from the Culex pipiens group

Thomas Walker¹, Lisa Klasson¹, Mohammed Sebaihia², Mandy J Sanders², Nicholas R Thomson², Julian Parkhill² and Steven P Sinkins*¹

Address: ¹Peter Medawar Building for Pathogen Research and Department of Zoology University of Oxford, South Parks Road, Oxford OX1 3PS, UK and ²Pathogen Sequencing Unit, Wellcome Trust Sanger Institute Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SA, UK

Email: Thomas Walker - thomas.walker@zoo.ox.ac.uk; Lisa Klasson - lisa.klasson@zoo.ox.ac.uk; Mohammed Sebaihia - ms5@sanger.ac.uk; Mandy J Sanders - mjs@sanger.ac.uk; Nicholas R Thomson - nrt@sanger.ac.uk; Julian Parkhill - parkhill@sanger.ac.uk; Steven P Sinkins* - steven.sinkins@zoo.ox.ac.uk

* Corresponding author

Abstract

**Background:** Wolbachia are obligate endosymbiotic bacteria maternally transmitted through the egg cytoplasm that are responsible for several reproductive disorders in their insect hosts, such as cytoplasmic incompatibility (CI) in infected mosquitoes. Species in the Culex pipiens complex display an unusually high number of Wolbachia-induced crossing types, and based on present data, only the wPip strain is present.

**Results:** The sequencing of the wPip strain of Wolbachia revealed the presence of 60 ankyrin repeat domain (ANK) encoding genes and expression studies of these genes were carried out in adult mosquitoes. One of these ANK genes, pk2, is shown to be part of an operon of three prophage-associated genes with sex-specific expression, and is present in two identical copies in the genome. Another homolog of pk2 is also present that is differentially expressed in different Cx. pipiens group strains. A further two ANK genes showed sex-specific regulation in wPip-infected Cx. pipiens group adults.

**Conclusion:** The high number, variability and differential expression of ANK genes in wPip suggest an important role in Wolbachia biology, and the gene family provides both markers and promising candidates for the study of reproductive manipulation.

Background

Wolbachia are obligate endosymbiotic bacteria that are maternally transmitted through the egg cytoplasm and are responsible for several reproductive disorders in arthropods, such as cytoplasmic incompatibility (CI) in infected Culex mosquitoes [1,2] and many other insects. Although Wolbachia are not found in mature sperm, they can modify developing sperm, possibly via chromatin binding proteins [3], such that when they fertilise an uninfected egg embryonic development is arrested. The reciprocal cross between infected females and uninfected males is, however, compatible; Wolbachia-infected females therefore produce a higher mean number of offspring than uninfected females. This unidirectional CI enables Wolbachia to rapidly invade uninfected populations [4], and provides a mechanism for driving anti-pathogen transgenes or a lifespan-shortening phenotype into mosquito populations [5,6]. Bidirectional CI can also occur between
insect populations, usually when they are infected with different strains of Wolbachia.

The genome sequence of the wMel strain [7], a CI-inducing Wolbachia strain found in Drosophila melanogaster, revealed an unusually high number of ankyrin repeat domain (ANK) encoding genes. Ankyrin repeats, consisting of around 33 residues, have been identified in a large number of proteins [8]. Ankyrin repeats are known to mediate protein-protein interactions in eukaryotes and are present in proteins involved in very different functions including cell cycle regulation, mitochondrial enzymes, cytoskeleton interactions, signal transduction and toxins [9]. Although ankyrin repeats are common in both eukaryotic and viral proteins they are relatively rare in bacteria and their function remains largely unknown. A protein containing ankyrin repeats in the bacterium Ehrlichia phagocytophila was detected in the host cytoplasm and found to be associated with chromatin suggesting a possible role in host cell gene expression [10]. ANK proteins have also been shown to mediate protein-protein interactions in cyclin-dependent kinase (CDK) inhibitors. In Nasonia wasps, the control of host cell cycle timing at karyogamy appears to be disrupted in CI and inhibition of CDK1 has been proposed as a possible mechanism [11,12]. Taken together this has led to the suggestion that ANK genes could play a role in Wolbachia-induced CI [7].

Species in the Cx. pipiens complex display an extremely high number of Wolbachia-induced crossing types between populations, with a high frequency of unidirectional incompatibilities [13-15]. Despite the complexity of crossing types, no polymorphism in the wPip strain of Wolbachia, responsible for CI in Cx. pipiens mosquitoes, has been found in the nucleotide sequences of ftsZ [15] and 16S rRNA [16] or in the highly variable wsp (surface protein) gene [17]. Sequencing of the wPip genome was undertaken partly in order to attempt to resolve this discrepancy. Interestingly, sequence analysis of some ANK genes found in wPip revealed variation in both nucleotide sequence and predicted amino acid sequence for two prophage associated ANK genes, pk1 and pk2, between wPip-infected Cx. pipiens colonies [17]. The wAu strain of Wolbachia, found in Drosophila simulans, is closely related to the wMel strain but does not normally induce CI [18]. The homolog of pk2 in wAu contains a premature stop codon not present in the wMel homolog, which suggests it could be a candidate gene for involvement in CI in Drosophila [19].

Variable expression between sexes and strains of Cx. pipiens was detected for the pk2 gene, a characteristic that might be expected for genes involved in the specific modification and rescue functions between incompatible strains. Any differential expression of ANK genes between male and female wPip infected adult Cx. pipiens mosquitoes would suggest an important function of these genes in the interaction between Wolbachia and its insect host. How Wolbachia differentially modify sperm in males as well as rescue in females is as yet unknown, but could potentially involve variability in the expression and activity of Wolbachia genes in male and female insect hosts. Variable gene expression in Wolbachia is not thought to occur at a high rate, as only a small number of regulatory genes have been identified in the Wolbachia genomes sequenced to date [20]. In this study, we analysed the expression profile of all ANK genes in wPip in Cx. pipiens adult mosquitoes.

**Results**

**Number and distribution of wPip ANK genes**

Analysis of the wPip genome revealed 60 ANK genes, which are numbered sequentially in Table 1. Several ANK proteins have predicted signal peptides and transmembrane domains. Thirteen of the wPip ANK genes are contained in several chromosomally integrated prophage regions, similar in sequence to the wMel WO-B prophage region [7]. The ANK genes pk1 and pk2, homologues of the wMel genes WD0596 and WD0636 respectively and previously shown to vary between incompatible Culex strains [17], are here shown to be present in multiple identical copies in different prophage regions: wPip_ANK8, wPip_ANK14 and wPip_ANK56 in the case of pk1 and wPip_ANK12 and wPip_ANK25 in the case of pk2. Two sequence variants of pk2 in wPip from different Cx. pipiens group colonies have been previously described and were named a and b. The wPip_ANK16 gene is also homologous to the pk2 genes/WD0636 in the wMel strain and is present in wPip in all the infected Cx. pipiens group colonies listed. A third pair of identical prophage-associated genes are also present, wPip_ANK13 and wPip_ANK26, which are homologues of WD0637. Thus in total there are 56 unique ANK genes present in the wPip genome.

Only 15 of the 23 wMel ANK genes have clear homologues in the wPip genome, which might reflect the high degree of heterogeneity in this group of genes. Thus, when likely paralogous groups (three non-identical homologues of WD0566 and two each of WD0636 and WD0637) and identical copies are taken into account, 37 of the 60 identified wPip ANK genes in the wPip genome do not have any clear homologues in the wMel genome. By way of comparison, the wBm strain of Wolbachia, thought to be a nutritional mutualist in the filarial nematode Brugia malayi, encodes only five ANK proteins [21], three of which are homologous to the wPip ANK encoding genes.
**Table 1: Ankyrin repeat domain encoding genes in the wPip genome**

| ANK gene | ANK repeats | Gene length (bp) | wMel homolog, wBm homolog | Additional gene information |
|----------|-------------|------------------|---------------------------|-----------------------------|
| wPip_ANK1 | 8           | 3324             | WD0147                    |                             |
| wPip_ANK2 | 1           | 675              |                           |                             |
| wPip_ANK3 | 2           | 1506             | WD0754                    | 2 transmembrane domains     |
| wPip_ANK4 | 2           | 1020             |                           | 2 transmembrane domains     |
| wPip_ANK5 | 4           | 1215             |                           | 2 transmembrane domains, DnaJ domain |
| wPip_ANK6 | 3           | 750              |                           | 2 transmembrane domains     |
| wPip_ANK7 | 3           | 642              |                           |                             |
| wPip_ANK8# | 8           | 1473             | WD0596                    | Prophage associated, 2 transmembrane domains |
| wPip_ANK9 | 10          | 8249             |                           |                             |
| wPip_ANK10| 4           | 5913             |                           |                             |
| wPip_ANK11| 2           | 1947             | WD0292                    | Prophage associated         |
| wPip_ANK12# | 3           | 450              | WD0636                    | Prophage associated         |
| wPip_ANK13* | 3           | 711              | WD0637                    | Prophage associated         |
| wPip_ANK14# | 8           | 1473             | WD0596                    | Prophage associated, 2 transmembrane domains |
| wPip_ANK15| 3           | 813              | WD0637                    | Prophage associated         |
| wPip_ANK16| 3           | 486              | WD0636                    | Prophage associated         |
| wPip_ANK17| 7           | 3102             | WD0636                    |                             |
| wPip_ANK18| 2           | 1026             | WD0636                    | DnaJ domain                 |
| wPip_ANK19| 2           | 498              | WD0566                    | 1 transmembrane domain      |
| wPip_ANK20| 11          | 2358             |                           |                             |
| wPip_ANK21| 4           | 1377             |                           |                             |
| wPip_ANK22| 7           | 2328             |                           |                             |
| wPip_ANK23| 2           | 7863             |                           |                             |
| wPip_ANK24| 12          | 2721             |                           |                             |
| wPip_ANK25# | 3           | 450              | WD0636                    | Prophage associated         |
| wPip_ANK26# | 3           | 711              | WD0637                    | Prophage associated         |
| wPip_ANK27| 2           | 534              | WD0566                    | Prophage associated, 1 transmembrane domain, signal peptide |
| wPip_ANK28| 5           | 7989             |                           |                             |
| wPip_ANK29| 7           | 912              | WD0766, Wbm0296           |                             |
| wPip_ANK30| 2           | 726              |                           |                             |
| wPip_ANK31| 8           | 1074             |                           |                             |
| wPip_ANK32| 4           | 546              |                           |                             |
| wPip_ANK33| 4           | 864              |                           | 2 transmembrane domains     |
| wPip_ANK34| 1           | 981              | WD0441, Wbm0582           | Signal peptide             |
| wPip_ANK35| 4           | 2049             | WD0438, Wbm0447           | 2 transmembrane domains     |
| wPip_ANK36| 10          | 1341             | WD0498/WD0499             | 1 transmembrane domain      |
| wPip_ANK37| 3           | 1779             |                           |                             |
| wPip_ANK38| 2           | 1146             |                           |                             |
| wPip_ANK39| 3           | 1119             |                           |                             |
| wPip_ANK40| 3           | 1170             |                           |                             |
| wPip_ANK41| 5           | 1182             |                           |                             |
| wPip_ANK42| 18          | 3411             |                           |                             |
| wPip_ANK43| 1           | 789              | WD0191                    | 2 transmembrane domains     |
| wPip_ANK44| 3           | 1389             |                           | 1 transmembrane domain      |
| wPip_ANK45| 3           | 1665             |                           | 2 transmembrane domains     |
| wPip_ANK46| 5           | 861              |                           |                             |
| wPip_ANK47| 11          | 2448             |                           | Signal peptide             |
| wPip_ANK48| 3           | 753              |                           | 2 transmembrane domains     |
| wPip_ANK49| 3           | 891              |                           | 2 transmembrane domains     |
| wPip_ANK50| 6           | 864              | WD0035                    |                             |
| wPip_ANK51| 1           | 978              |                           |                             |
| wPip_ANK52| 12          | 1977             | WD0385                    |                             |
| wPip_ANK53| 2           | 1302             |                           | 1 transmembrane domain      |
| wPip_ANK54| 6           | 1158             |                           | 2 transmembrane domains     |
| wPip_ANK55| 3           | 1983             | WD0633                    | Prophage associated         |
| wPip_ANK56# | 8           | 1473             | WD0596                    | Prophage associated, 2 transmembrane domains |
| wPip_ANK57| 2           | 519              | WD0566                    | Prophage associated, 1 transmembrane domain |
| wPip_ANK58| 3           | 3687             |                           |                             |
| wPip_ANK59| 7           | 1152             |                           |                             |
| wPip_ANK60| 8           | 1695             |                           |                             |

The number of ANK domains as identified by Pfam, gene length (bp), the wMel and wBm homologous gene where there this can be clearly determined. Symbols # (pk1), * (pk2) and + denote groups of prophage-associated genes with identical sequences.
**ANK gene expression**
Transcripts were detected for all of the ANK encoding genes. For the majority, expression in adult males and females of the Pel colony was not obviously different based on agarose gel electrophoresis of RT-PCR products. The *wPip_ANK57* gene showed very low expression in Pel female extracts and no detectable expression in Pel male RNA extracts. *wPip_ANK2* and *wPip_ANK49* showed low levels of expression in both Pel male and female RNA extracts. RT-PCR analysis also suggested that *wPip_ANK38* is highly expressed in both sexes.

**wPip_ANK12 and wPip_ANK25**
The identical prophage associated ANK encoding genes *wPip_ANK12* and *wPip_ANK25*, previously together named *pk2* [17], showed the greatest difference in expression between sexes, with no detectable RT-PCR products in the males of the Pel and Mol colonies. Expression of these genes was also not detected in males for an additional *Cx. pipiens* colony from Sri Lanka (Sumo Cypppe). Quantification of expression by quantitative reverse transcription (qRT-PCR) was carried out and the mean male expression of the *pk2* gene in the Pel colony in comparison to female expression was 1.6% (Figure 1). However, expression of *pk2* was observed at similar levels in males and females of the Col colony. Primers were designed to discriminate between *pk2* sequence variants *pk2a* present in the Pel, Sumo and Mol colonies and *pk2b* present in the Col colony and confirmed no detectable expression of *pk2a* from male RNA extracts of the Pel, Sumo Cypppe and Mol colonies using RT-PCR (Figure 2). The *pk2b* gene variant was expressed at similar levels in Col colony adult females and males. Further RT-PCR analysis showed *pk2* gene expression in both preblastoderm embryos and pooled 4th instar larvae (sex undetermined) of the Pel colony. *pk2* expression in pooled testes from 20 Pel males was just detectable but the RT-PCR product was very weak compared to those for *wsp* and *pk1* (not shown).

Differential expression between sexes was also observed for two genes directly downstream of *pk2* (Figure 3). *pk2-1* encodes a hypothetical protein present in identical copies in the two *pk2* associated prophage regions. *pk2-2* encodes a site-specific recombinase present in almost identical copies in the two prophage regions. Primers used for expression studies could not discriminate between the *pk2-2* copies. For the gene upstream of *pk2*, also an ANK encoding gene present in two identical copies (*wPip_ANK13* and *wPip_ANK26*), RT-PCR followed by agarose gel electrophoresis revealed similar expression levels in both female and male RNA extracts of the Pel colony. Primers designed to span the intergenic regions of *pk2/pk2-1/pk2-2* produced RT-PCR products from females but no detectable products from males of the Pel colony. Primers spanning the intergenic region between *pk2* and *pk2+1* (primers 5 and 6) produced no amplification of a transcript from either female or male RNA extracts of the Pel colony. However, using the same primers, a product of correct size (733 bp) was amplified in both male and female DNA extracts of the Pel colony.

**Figure 1**
ANK gene expression in adult *wPip*-infected Pel males (*Cx. pipiens*). The mean ± SE for expression in individual adult males (6) in comparison to expression in females is shown after normalization using the *wsp* gene.

**wPip_ANK16**
The *pk2/WD0636* homolog *wPip_ANK16* was present in all the *Culex* strains tested based on PCR amplification. In the Pel, Mol and Sumo Cypppe colonies, *wPip_ANK16* was expressed equally in males and females; however for the Col colony no expression could be detected in males, and only a weak RT-PCR product could be detected in females (Figure 2).

**wPip_ANK1**
Standard RT-PCR analysis followed by agarose gel electrophoresis revealed much lower expression levels of *wPip_ANK1* from pooled male RNA extracts of all *wPip*-infected *Culex* colonies in comparison to female RNA extracts. Quantification of expression by qRT-PCR was undertaken and the mean male normalized expression of the *wPip_ANK1* gene in Pel males relative to Pel female expression was 4.7% (Figure 1). Expression levels were similar in Pel females and males for both genes flanking *wPip_ANK1*, based on standard RT-PCR followed by agarose gel electrophoresis.
Expression analysis using RT-PCR followed by gel electrophoresis revealed lower expression levels of \( \text{wPip}_\text{ANK52} \) from pooled male RNA extracts of all \( \text{wPip} \)-infected \( \text{Cx. pipiens} \) colonies in comparison to female RNA extracts. Quantification of expression by qRT-PCR was undertaken and the mean male normalized expression of the \( \text{wPip}_\text{ANK52} \) gene in Pel males relative to Pel female expression was 19% (Figure 1). Reduced expression levels were also observed in Pel male RNA extracts for three additional genes flanking \( \text{wPip}_\text{ANK52} \) based on standard RT-PCR followed by agarose gel electrophoresis. However, although standard PCR using primers to span the intergenic regions of these genes resulted in an amplified product of approximately 1.5 Kb, no products were amplified using RT-PCR from either Pel female or male RNA extracts.

**Discussion**

The presence of 60 ANK genes is significantly more than the 23 identified in the \( \text{wMel} \) genome [7]; in fact, the number and density of ANK genes is the highest reported for any prokaryotic genome. The expansion of ANK genes in the \( \text{wPip} \) strain, the degree of sequence variability and sex-specific expression in adult \( \text{Cx. pipiens} \) mosquitoes suggests an important biological role in parasitic strains of \( \text{Wolbachia} \). The RT-PCR analysis provides strong evidence for a single transcriptional unit (operon) produced from three prophage associated genes including \( \text{pk}2 \). However, there was no evidence that \( \text{wPip}_\text{ANK1} \) and \( \text{wPip}_\text{ANK52} \) are part of sex-specifically regulated operons. The quantitative RT-PCR analysis in this study represents only an estimation of differences in relative ANK gene expression. The accurate quantification of RNA expression in bacteria has been limited due to the absence of reliable standardization. In eukaryotic cells, stably expressed housekeeping genes can be used as standards to perform relative quantification of gene expression. For an endosymbiotic bacterium such as \( \text{Wolbachia} \), comparing the expression of ankyrin genes to the surface protein encoding gene (\( \text{wsp} \)) was used to normalize for variation in \( \text{Wolbachia} \) density but any differences in levels of \( \text{wsp} \) expression between sexes and stages could be a confounding factor. The \( \text{wsp} \) gene was previously shown to be expressed in all \( \text{Cx. pipiens} \) life stages including male and female adults [22].

As some of the ANK proteins have predicted signal peptides and transmembrane domains, it is possible that they are secreted into the mosquito cytoplasm or presented on the surface of the bacterium, which could suggest that they

| Gene variant | Primer sequences 5'-3' | PCR product size |
|--------------|------------------------|------------------|
| \( \text{pk2} \) 'a' | F: CCGCGAACGTCTAGAATCG R: TCTGCTATTTCCTTCTTC | 245 bp |
| \( \text{pk2} \) 'b' | F: AGGGATCTAAATGGCGAAG R: CCTGCTGTACCTCCTTCTTTT | 202 bp |
| \( \text{wPip}_\text{ANK16} \) | F: GAGACGAGAATGGAGAACGACG C: CTTCATTTGTCTGCTGCTTTC | 286 bp |

**Figure 2**

*pk2* gene variants/homologs in \( \text{wPip} \)-infected \( \text{Cx. pipiens} \) group colonies. The primer sequences (5'-3') and expression levels are shown resulting from RT-PCR analysis of female (F) and male (M) RNA extracts of adult \( \text{wPip} \)-infected adults.
are involved in Wolbachia’s interaction with the host. A proteomics analysis including experiments such as immunolocalisation studies could be used to characterize the function of ANK proteins in Wolbachia. Current limitations to such studies include the difficulty of obtaining epitope specificity and the absence of a transformation system for Wolbachia. As co-regulated genes are highly likely to show functional interactions, studies to examine the role of the co-expressed prophage-associated genes adjacent to pk2 are also needed.

Associations between ANK gene sequence variants and particular crossing types have previously been reported [17], enabling use of these variants as markers to further investigate Wolbachia-induced CI in the Cx. pipiens group. The significance of sex-specific expression patterns in the pk2 genes in some host strains but not others is not yet understood, but its occurrence did not correlate with the crossing patterns described in Table 2. The Mol and Pel colonies are bidirectionally incompatible with each other but both show the same sex-specific expression of the pk2 genes in adult mosquitoes. Given the complexity of the phenotype in the Cx. pipiens group, it seems plausible or even probable that the genetic basis for these crossing type differences involves multiple Wolbachia genes, and factors such as the mosquito nuclear background interacting with Wolbachia variants can also contribute [17]. A hypothesis that variation at just one ‘CI gene’ could explain all the crossing type variation observed seems increasingly unlikely. Given the rapid evolution of ANK genes, sequence differences at particular ANK loci between crossing types does not necessarily mean that there is a causal link. However the differential expression between sexes of several ANK genes (including non prophage-associated genes) in wPip does provide further support for adaptations to sex-specific interactions with its host.

### Conclusion

The number of ANK genes in the wPip genome is the highest yet reported in a prokaryote. The sex-specificity observed in patterns of expression for some of these genes and the differential expression between mosquito strains are also very unusual features, particularly given the generally very high level of sequence conservation between wPip variants. The elucidation of the functional roles and mechanisms of evolution of this family of genes will provide many insights into the biology of reproductive parasites.

### Table 2: Percentage embryo hatch in crosses between the colonies, using 50 individuals of each sex and counting hatch rates of a minimum of eight individual egg rafts

|                | Pel male | Col male | Mol male |
|----------------|----------|----------|----------|
| Pel female     | -        | 72.92 ± 0.96 | 0.17 ± 0.12 |
| Col female     | 44.33 ± 2.39 | -        | 0.44 ± 0.19 |
| Mol female     | 0 ± 0    | 93.49 ± 1.17 | -        |

---

**Figure 3**

Sex-specific expression in adult Pel colony mosquitoes for a prophage operon containing the pk2 gene. The RT-PCR expression profile for male (M) and female (F) RNA extracts is shown for each gene in addition to RT-PCR products generated by using primers (P1–P6) designed to amplify fragments spanning the intergenic regions.
Methods

Identification of ANK genes and primer design
Putative protein-encoding genes were identified in the wPip genome using ORPHEUS [23], followed by manual curation. The translated gene sequences were searched against the Interpro database using Interproscan [24] in order to locate ankyrin repeats and other protein motifs such as signal peptides and transmembrane domains. The protein sequences containing ANK domains were compared to the protein sequences of Wolbachia strain wMel using blastp in order to identify possible homologs. Identification of a putative origin of replication and the assignment of ANK gene numbers was based on the location of the dnaA gene. Gene specific primers with an annealing temperature ranging between 50–55°C were designed for all unique ANK genes (Additional file 1) using Primer Select 5.06 (DN Aster, Madison, WI, USA) and Primer3 [25]. The unfinished sequence of the wPip genome and the corresponding preliminary annotation of the ANK genes are available from the Wellcome Trust Sanger Institute website, and will be updated as the sequence is completed [26].

Mosquito colonies
Colonies of wPip-infected Cx. pipiens mosquitoes were selected for the study. Table 3 lists the colonies used in addition to the location of where the colonies originated. All mosquito colonies were reared using standard rearing procedures at low larval densities in insectary conditions (26°C and 70% relative humidity) with a 12:12 h light/dark circadian cycle. Mass crossing experiments between Cx. molestus males and Cx. pipiens females were carried out under a dissecting microscope in 0.1% saline after immobilising adult mosquitoes on ice. Dissected testes were rinsed in PBS and then pooled in 1.5 mL microcentrifuge tubes in RNA later (Ambion, Austin, TX, USA) to prevent RNA degradation. The quality and yield of total RNA was measured using a Nanodrop ND 100 spectrophotometer.

RNA extraction
Total RNA was extracted from young (1–2 days post eclosion) adult mosquitoes using Tri Reagent (Sigma-Aldrich) followed by chloroform extraction and isopropanol precipitation. RNA extracts were treated with DNase I (Sigma-Aldrich) to remove any contaminating DNA. As the density of Wolbachia is significantly lower in adult male Culex mosquitoes compared to females, three adult Cx. pipiens male mosquitoes were pooled prior to RNA extraction to increase the amount of Wolbachia RNA present for analysis. RNA extraction of testes was carried out by dissection of 20 Pel colony males under a dissecting microscope in 0.1% saline after immobilising adult mosquitoes on ice. Dissected testes were rinsed in PBS and then pooled in 1.5 mL microcentrifuge tubes in RNA later (Ambion, Austin, TX, USA) to prevent RNA degradation. PCR assays were optimised by testing at numerous annealing temperatures.

RT-PCR
Reverse transcription (RT) PCR analysis was performed using the Qia gen One step RT-PCR kit (Hilden, Germany). RNase-free water, One step RT-PCR buffer (1×), 400 µM dNTPs and One step RT-PCR enzyme mix were combined with gene specific primers (0.6 µM) to amplify 2.0 µL of template RNA in 50 µL reactions. Reverse transcription was carried out at 50°C for 30 min followed by 95°C for 15 min. Samples were PCR amplified by denaturing for 5 min at 94°C, cycled 35 times at 94°C (1 min) variable annealing temperature (1 min) and 72°C (1 min each), followed by a 10 min extension at 72°C using an Applied Biosystems GeneAmp PCR system 2700. A total of 10 µL of RT-PCR products and a 100 base-pair marker (Sigma-Aldrich) was electrophoresed on 1% agarose gels stained with ethidium bromide and visualized under ultraviolet illumination. To examine for false positives that might result from amplification of DNA, parallel reactions without adding the reverse transcriptase (Taq polymerase only, Sigma-Aldrich) to the reaction mixture were included.

Quantitative RT-PCR
Quantification of gene expression was carried out using the Qiagen One step SYBR green RT-PCR kit and the Opticon 2 Continuous Fluorescence Detection System.

Table 3: List of mosquitoes used in the study with the colony/strain in addition to the origin where the colony/strain was first obtained

| Mosquito species           | Colony/strain | Origin     |
|----------------------------|---------------|------------|
| Culex quinquefasciatus     | Pel           | Sri Lanka  |
| Culex quinquefasciatus     | Col           | Colombia   |
| Culex molestus             | Mol           | China      |
Primers were designed to amplify ANK gene fragments of less than 250 bp. Standard curves were produced using serial dilution of RNA extracted from adult female mosquitoes and relative male RNA extract expression of ANK genes measured in comparison. Quantitative RT-PCR cycling conditions were 50°C for 30 min followed by 95°C for 15 min. Samples were cycled 40 times at 94°C (15 s), 55°C (30 s) and 72°C (30 s) followed by a read step. A melting curve was constructed between 50°C and 90°C. Quantitative RT-PCR assays were carried out on six male RNA extracts in two separate assays. Comparing the concentration of cDNA amplified from ankyrin genes to the wsp gene was used for normalization of the data, to control for both differences in extraction efficiency and also the higher Wolbachia density that occurs in adult female mosquitoes compared to males. The mean relative expression levels of the wsp gene in Pel males, used to normalize for differential Wolbachia density in individual adult mosquitoes, was found to be 44.2 ± 9.6% compared to expression levels in Pel female RNA extracts.

Competing interests
The author(s) declares that there are no competing interests.

Authors’ contributions
TW designed and conducted expression experiments and analyses, LK contributed to primer design, genome analyses and annotation, MS, MJS, NT and JP carried out genome analyses and assembly, and SPS contributed experimental design, co-ordination and analyses. TW, LK and SPS wrote the paper and all authors read and approved the manuscript.

Additional material
Additional file 1
wPlp ANK gene primers. ANK gene primers, optimal PCR annealing temperatures and PCR product sizes used for reverse transcription PCR analysis. Identical prophage-associated genes at different locations in the genome are listed together. Click here for file [http://www.biomedcentral.com/content/supplementary/1741-7007-5-39-S1.doc]

Acknowledgements
This work was funded by the Wellcome Trust. SPS is a Wellcome Trust Senior Research Fellow in Basic Biomedical Science, TW was also supported by an award from the Sir Halley Stewart Trust, and LK is an EU Marie Curie Fellow.

References
1. Laven H: Crossing experiments with Culex strains. Evolution 1951, 5:370-375.
2. Barr AR: Cytoplasmic incompatibility as a means of eradication of Culex pipiens L. Proc Pap Ann Conv Calif Mosq Control Assoc 1966, 34:22-35.
3. McGraw EA, O’Neill SL: Wolbachia pipientis: intracellular infection and pathogenesis in Drosophila. Curr Opin Microbiol 2004, 7:67-70.
4. Turelli M, Hoffmann AA: Rapid spread of an inherited incompatibility factor in California Drosophila. Nature 1991, 353:440-442.
5. Sinkins SP, O’Neill SL: Wolbachia as a vehicle to modify insect populations. In Insect Transgenesis: Methods and Applications Edited by: Handler AM, James AA. Boca Raton: CRC Press; 2000:271-288.
6. Dobson SL: Reversing Wolbachia-based population replacement. Trends Parasitol 2003, 19:128-133.
7. Wu M, Sun LV, Yamatehavan J, Riegler M, Deboy R, Brownie JC, McGraw EA, Martin W, Esser C, Ahmadnejad N, Wiegand C, Madupu R, Beanan MJ, Brinklak LM, Duagherty SC, Durkin AS, Kolo-
8. McCallum BB, Nelson WC, Mohamow J, Lee P, Berry E, Yeates MB, Utter-
9. Back T, Weidman J, Nierman WC, Paulsen IT, Nelson KE, Tettelin H, Lavelle SP, Eisen JA: Phylogenomics of the Reproductive Para-
10. Wolbachia pipientis wMel: A Streamlined Genome Overrun by Mobile Genetic Elements. PLoS Biol 2004, 2:e59.
11. Mosavi K, Cammert TJ, Desrosiers DC, Peng ZY: The ankyrin repeat as molecular architecture for protein recognition. Protein Sci 2004, 13:1435-1448.
12. Sedgwick SG, Smerdon SJ: The ankyrin repeat: a diversity of interactions on a common structural framework. Trends Bio-
13. chemistry 1999, 24:311-316.
14. Subbaramo SK: Cytoplasmic incompatibility in mosquitoes. In Recent Developments in the Genetics of Insect Disease Vectors Edited by: Steiner VWM, Tabachnick WJ, Rai KS, Narang S. Champaign: Stipes; 1992:313-342.
15. Maguin M, Pasteur N, Raymond M: Multiple incompatibilities within populations of Culex pipiens L. in southern France. Genetica 1987, 74:125-130.
16. Guillemaud T, Pasteur N, Rousset F: Contrasting levels of varia-
17. bility between cytoplasmic genomes and incompatibility types in the mosquito Culex pipiens. Proc R Soc Lond B Biol Sci 1997, 264:245-251.
18. Stouthamer R, Breeuwer JA, Luck RF, Werren JH: Molecular identi-
19. fication of microorganisms associated with parthenogene-
20. sis. Nature 1993, 361:66-68.
21. Sinkins SP, Walker T, Lynd AR, Steven AR, Makepeace BL, Godfray HC, Parkhill J: Wolbachia variability and host effects on cross-
22. ing type in Culex mosquitoes. Nature 2005, 436:257-260.
23. Mercot H, Charlat S: Wolbachia infections in Drosophila melan-
24. ogaster and D. simulans: polymorphism and levels of cyto-
25. plasmic incompatibility. Genetics 2004, 120:51-59.
26. Iturbe-Ormaetxe I, Burke GR, Riegler M, O’Neill SL: Distribution, Expression, and Motif Variability of Ankyrin Domain Genes in Wolbachia pipientis. J Bacterial 2005, 187:5136-5145.
27. Fenon K, Blaxter M: Wolbachia genomes: revealing the biology of parasitism and mutualism. Trends Parasitol 2006, 22:60-65.
28. Foster J, Ganatra M, Kamal I, Ware J, Makarova K, Ivanova N, Bhattacharyya A, Kapralov V, Kumar S, Postfai J, Vincze T, Ingrain J, Moran L, Lapidus A, Omelchenko M, Kyrpides N, Ghedin E, Wang S, Goltsman E, Jukov V, Oztsovkaya O, Tsukerman K, Mazur M, Comb D, Koonin E, Slatko B: Repeat as molecular architecture for protein recognition. Protein Sci 2004, 13:1435-1448.
29. Sedgwick SG, Smerdon SJ: The ankyrin repeat: a diversity of interactions on a common structural framework. Trends Bio-
30. chemistry 1999, 24:311-316.
31. Subbaramo SK: Cytoplasmic incompatibility in mosquitoes. In Recent Developments in the Genetics of Insect Disease Vectors Edited by: Steiner VWM, Tabachnick WJ, Rai KS, Narang S. Champaign: Stipes; 1992:313-342.
32. Maguin M, Pasteur N, Raymond M: Multiple incompatibilities within populations of Culex pipiens L. in southern France. Genetica 1987, 74:125-130.
33. Guillemaud T, Pasteur N, Rousset F: Contrasting levels of varia-
34. bility between cytoplasmic genomes and incompatibility types in the mosquito Culex pipiens. Proc R Soc Lond B Biol Sci 1997, 264:245-251.
35. Stouthamer R, Breeuwer JA, Luck RF, Werren JH: Molecular identi-
36. fication of microorganisms associated with parthenogene-
37. sis. Nature 1993, 361:66-68.
38. Sinkins SP, Walker T, Lynd AR, Steven AR, Makepeace BL, Godfray HC, Parkhill J: Wolbachia variability and host effects on cross-
39. ing type in Culex mosquitoes. Nature 2005, 436:257-260.
40. Mercot H, Charlat S: Wolbachia infections in Drosophila melan-
41. ogaster and D. simulans: polymorphism and levels of cyto-
42. plasmic incompatibility. Genetics 2004, 120:51-59.
43. Iturbe-Ormaetxe I, Burke GR, Riegler M, O’Neill SL: Distribution, Expression, and Motif Variability of Ankyrin Domain Genes in Wolbachia pipientis. J Bacterial 2005, 187:5136-5145.
44. Fenon K, Blaxter M: Wolbachia genomes: revealing the biology of parasitism and mutualism. Trends Parasitol 2006, 22:60-65.
45. Foster J, Ganatra M, Kamal I, Ware J, Makarova K, Ivanova N, Bhattacharyya A, Kapralov V, Kumar S, Postfai J, Vincze T, Ingrain J, Moran L, Lapidus A, Omelchenko M, Kyrpides N, Ghedin E, Wang S, Goltsman E, Jukov V, Oztsovkaya O, Tsukerman K, Mazur M, Comb D, Koonin E, Slatko B: Repeat as molecular architecture for protein recognition. Protein Sci 2004, 13:1435-1448.
sequenced bacterial genomes. Nucleic Acids Res 1998, 26:2941-2947.
24. Zdobnov EM, Apweiler R: InterProScan – an integration platform for the signature-recognition methods in InterPro. Bioinformatics 2001, 17:847-848.
25. Rozen S, Skaletsky H: Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000, 132:365-386.
26. Wolbachia pipientis endosymbiont of Culex quinquefasciatus [http://www.sanger.ac.uk/Projects/W_pipientis/]
27. Collins FH, Mendez MA, Rasmussen MO, Mehaffey PC, Besansky NJ, Finnerty V: A ribosomal RNA gene probe differentiates mem-
ber species of the Anopheles gambiae complex. Am J Trop Med Hyg 1987, 37:37-41.