

Research article

Open Access

Ankyrin repeat domain-encoding genes in the wPip strain of *Wolbachia* from the *Culex pipiens* group

Thomas Walker¹, Lisa Klasson¹, Mohammed Sebahia², 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 Sebahia - 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

Published: 20 September 2007

Received: 27 July 2007

BMC Biology 2007, 5:39 doi:10.1186/1741-7007-5-39

Accepted: 20 September 2007

This article is available from: <http://www.biomedcentral.com/1741-7007/5/39>

© 2007 Walker et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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 uni- or bidirectional 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		2 transmembrane domains
wPip_ANK3	2	1506	WD0754	
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		1 transmembrane domain
wPip_ANK18	2	1026		Dnaj domain
wPip_ANK19	2	498	WD0566	1 transmembrane domain
wPip_ANK20	11	2358		
wPip_ANK21	4	1377		2 transmembrane domains
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		2 transmembrane domains
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		2 transmembrane domains
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		2 transmembrane domains, Dnaj domain
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 Cyppe). 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 Cyppe 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 *usp* 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

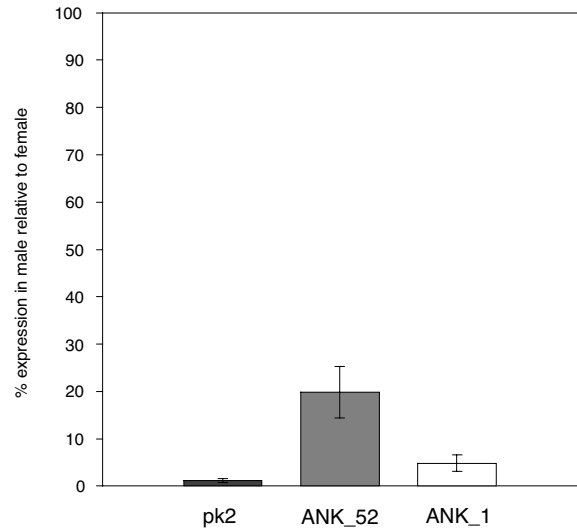


Figure 1

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

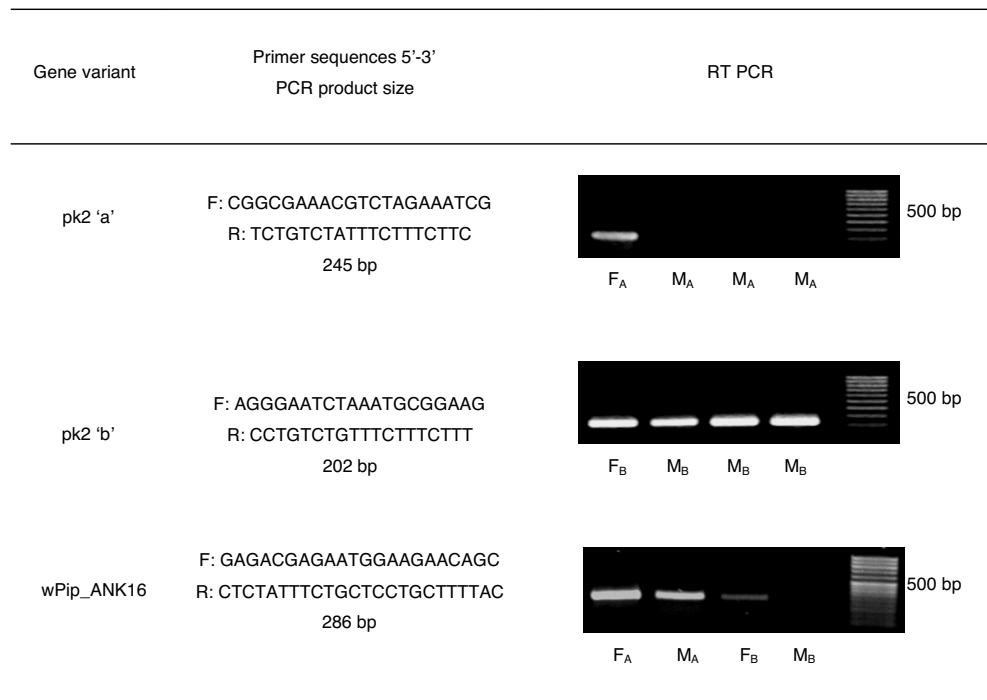
pk2+1 (primers 5 and 6) produced no amplification of a transcript from either female or male RNA extracts of the Pel colony. However, using the same primers, a product of correct size (733 bp) was amplified in both male and female DNA extracts of the Pel colony.

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 Cyppe 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.

**Figure 2**

pk2 gene variants/homologs in wPip-infected *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 wPip-infected adults.

wPip_ANK52

Expression analysis using RT-PCR followed by gel electrophoresis revealed lower expression levels of wPip_ANK52 from pooled male RNA extracts of all wPip-infected *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 wPip_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 wPip_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 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 wPip strain, the degree of sequence variability and sex-specific expression in adult *Cx. pipiens* mosquitoes suggests an important biological role in parasitic strains of

Wolbachia. The RT-PCR analysis provides strong evidence for a single transcriptional unit (operon) produced from three prophage associated genes including *pk2*. However, there was no evidence that wPip_ANK1 and wPip_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 *Wolbachia*, comparing the expression of ankyrin genes to the surface protein encoding gene (*wsp*) was used to normalize for variation in *Wolbachia* density but any differences in levels of *wsp* expression between sexes and stages could be a confounding factor. The *wsp* gene was previously shown to be expressed in all *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

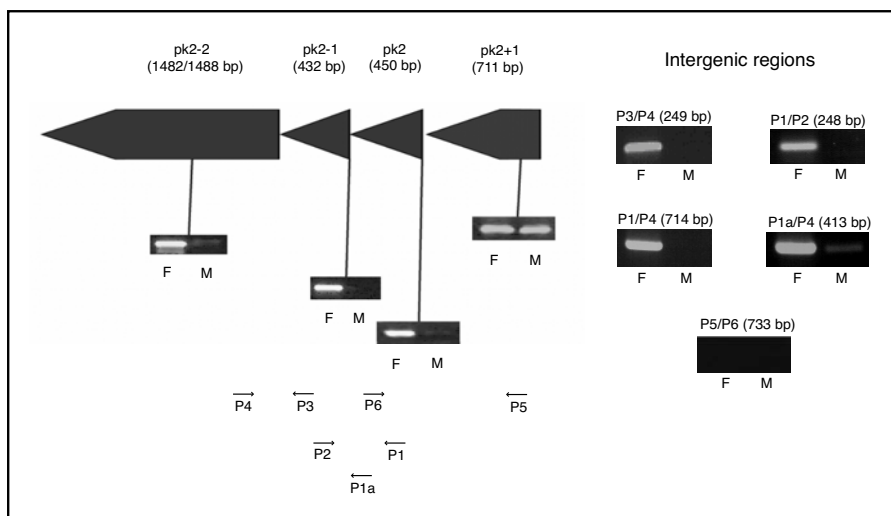


Figure 3
Sex-specific expression in adult Pel colony mosquitoes for a prophage operon containing the *pk2* gene. The RT-PCR expression profile for male (M) and female (F) RNA extracts is shown for each gene in addition to RT-PCR products generated by using primers (P1–P6) designed to amplify fragments spanning the intergenic regions.

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	-

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 (DNASTar, 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. pipiens* colonies were carried out using 50 virgin individuals of each sex. The F₁ generation progeny from the crosses was analysed by calculating the percentage of hatched embryos from a minimum of eight egg rafts, each containing between 50–110 eggs per raft, as a measure of the CI phenotype. Female spermathecae were examined for the presence of sperm if the hatch rate was low to confirm insemination.

PCR

All ANK gene primers were tested on Pel genomic DNA extracted using a modified version of the Livak buffer method with ethanol precipitation [27]. Genomic DNA was PCR amplified in 2.5 mM MgCl₂, 0.25 mM dNTPs,

0.5 μM forward and reverse primers, 0.2 units of *Taq* polymerase (Sigma-Aldrich, St Louis, MO, USA), *Taq* polymerase buffer (1×) and filter-sterilised water in a total volume of 20 μL. Standard PCR cycling conditions involved denaturing the samples for 5 min at 94°C, variable annealing temperature and 72°C (1 min each), followed by a 10 min extension at 72°C using an Applied Biosystems GeneAmp PCR system 2700 (Foster City, CA, USA). PCR assays were optimised by testing at numerous annealing temperatures.

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 RNAlater (Ambion, Austin, TX, USA) to prevent RNA degradation. The quality and yield of total RNA was measured using a Nanodrop ND 100 spectrophotometer.

RT-PCR

Reverse transcription (RT) PCR analysis was performed using the Qiagen Onestep RT-PCR kit (Hilden, Germany). RNase-free water, Onestep RT-PCR buffer (1×), 400 μM dNTPs and Onestep 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 Onestep 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
<i>Culex quinquefasciatus</i>	Pel	Sri Lanka
<i>Culex quinquefasciatus</i>	Col	Colombia
<i>Culex molestus</i>	Mol	China

(Genetic Research Instrumentation, Braintree, Essex, UK). 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 *usp* 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 *usp* gene in Pel males, used to normalize for differential *Wolbachia* density in individual adult mosquitoes, was found to be $44.2 \pm 9.6\%$ 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

wPip 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

- Laven H: **Crossing experiments with *Culex* strains.** *Evolution* 1951, **5**:370-375.
- Barr AR: **Cytoplasmic incompatibility as a means of eradication of *Culex pipiens* L.** *Proc Pap Annu Conf Calif Mosq Control Assoc* 1966, **34**:32-35.
- McGraw EA, O'Neill SL: ***Wolbachia pipientis*: intracellular infection and pathogenesis in *Drosophila*.** *Curr Opin Microbiol* 2004, **7**:67-70.
- Turelli M, Hoffmann AA: **Rapid spread of an inherited incompatibility factor in California *Drosophila*.** *Nature* 1991, **353**:440-442.
- 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.
- Dobson SL: **Reversing *Wolbachia*-based population replacement.** *Trends Parasitol* 2003, **19**:128-133.
- Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadijad N, Wiegand C, Madupu R, Beanan MJ, Brinkac LM, Daugherty SC, Durkin AS, Kolonay JF, Nelson WC, Mohamoud Y, Lee P, Berry K, Young MB, Utterback T, Weidman J, Nierman WC, Paulsen IT, Nelson KE, Tettelin H, O'Neill SL, Eisen JA: **Phylogenomics of the Reproductive Parasite *Wolbachia pipientis* wMel: A Streamlined Genome Overrun by Mobile Genetic Elements.** *PLoS Biol* 2004, **2**:E69.
- Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY: **The ankyrin repeat as molecular architecture for protein recognition.** *Protein Sci* 2004, **13**:1435-1448.
- Sedgwick SG, Smerdon SJ: **The ankyrin repeat: a diversity of interactions on a common structural framework.** *Trends Biochem Sci* 1999, **24**:311-316.
- Caturegli P, Asanovich KM, Walls JJ, Bakken JS, Madigan JE, Popov VL, Dumler JS: **ankA: an Ehrlichia phagocytophila group gene encoding a cytoplasmic protein antigen with ankyrin repeats.** *Infect Immun* 2000, **68**:5277-5283.
- Tram U, Ferree PM, Sullivan W: **Identification of *Wolbachia* – host interacting factors through cytological analysis.** *Microbes Infect* 2003, **5**:999-1011.
- Tram U, Sullivan W: **Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility.** *Science* 2002, **296**:1124-1126.
- Subbarao SK: **Cytoplasmic incompatibility in mosquitoes.** In *Recent Developments in the Genetics of Insect Disease Vectors* Edited by: Steiner WW, Tabachnick WJ, Rai KS, Narang S. Champaign: Stipes; 1982:313-342.
- Magnin M, Pasteur N, Raymond M: **Multiple incompatibilities within populations of *Culex pipiens* L. in southern France.** *Genetica* 1987, **74**:125-130.
- Guillemaud T, Pasteur N, Rousset F: **Contrasting levels of variability between cytoplasmic genomes and incompatibility types in the mosquito *Culex pipiens*.** *Proc R Soc Lond B Biol Sci* 1997, **264**:245-251.
- Stouthamer R, Breeuwer JA, Luck RF, Werren JH: **Molecular identification of microorganisms associated with parthenogenesis.** *Nature* 1993, **361**:66-68.
- Sinkins SP, Walker T, Lynd AR, Steven AR, Makepeace BL, Godfray HC, Parkhill J: ***Wolbachia* variability and host effects on crossing type in *Culex* mosquitoes.** *Nature* 2005, **436**:257-260.
- Mercot H, Charlat S: ***Wolbachia* infections in *Drosophila melanogaster* and *D. simulans*: polymorphism and levels of cytoplasmic incompatibility.** *Genetica* 2004, **120**:51-59.
- Iturbe-Ormaetxe I, Burke GR, Riegler M, O'Neill SL: **Distribution, Expression, and Motif Variability of Ankyrin Domain Genes in *Wolbachia pipientis*.** *J Bacteriol* 2005, **187**:5136-5145.
- Fenn K, Blaxter M: ***Wolbachia* genomes: revealing the biology of parasitism and mutualism.** *Trends Parasitol* 2006, **22**:60-65.
- Foster J, Ganatra M, Kamal I, Ware J, Makarova K, Ivanova N, Bhattacharyya A, Kapatral V, Kumar S, Posfai J, Vincze T, Ingram J, Moran L, Lapidus A, Omelchenko M, Kyrpidis N, Ghedin E, Wang S, Goltsman E, Joukov V, Ostrovskaya O, Tsukerman K, Mazur M, Comb D, Koonin E, Slatko B: **The *Wolbachia* Genome of *Brugia malayi*: Endosymbiont Evolution within a Human Pathogenic Nematode.** *PLoS Biol* 2005, **3**:e121.
- Sanogo YO, Dobson SL: **WO bacteriophage transcription in *Wolbachia*-infected *Culex pipiens*.** *Insect Biochem Mol Biol* 2006, **36**:80-85.
- Frishman D, Mironov A, Mewes HW, Gelfand M: **Combining diverse evidence for gene recognition in completely**

- 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 member species of the Anopheles gambiae complex.** *Am J Trop Med Hyg* 1987, **37**:37-41.

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

