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Variability and Expression of Ankyrin Domain Genes in *Wolbachia* Variants Infecting the Mosquito *Culex pipiens*▽†

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***Wolbachia* strains are maternally inherited endosymbiotic bacteria that infect many arthropod species and have evolved several different ways of manipulating their hosts, the most frequent way being cytoplasmic incompatibility (CI). CI leads to embryo death in crosses between infected males and uninfected females as well as in crosses between individuals infected by incompatible *Wolbachia* strains. The mosquito *Culex pipiens* exhibits the highest crossing type variability reported so far. Our crossing data support the notion that CI might be driven by at least two distinct genetic units that control the CI functions independently in males and females. Although the molecular basis of CI remains unknown, proteins with ankyrin (ANK) domains represent promising candidates since they might interact with a wide range of host proteins. Here we searched for sequence variability in the 58 ANK genes carried in the genomes of *Wolbachia* variants infecting *Culex pipiens*. Only five ANK genes were polymorphic in the genomes of incompatible *Wolbachia* variants, and none correlated with the CI pattern obtained with 15 mosquito strains (representing 14 *Wolbachia* variants). Further analysis of ANK gene expression evidenced host- and sex-dependent variations, which did not improve the correlation. Taken together, these data do not support the direct implication of ANK genes in CI determinism.**

Wolbachia spp. are maternally inherited alpha proteobacteria that are widespread among filarial nematodes and arthropods and that infect many insect species (35, 36, 39). The successful spread of *Wolbachia* spp. is attributed to their ability to alter host reproduction to their own advantage by inducing the feminization of genetic males, male killing, parthenogenesis, and most commonly, cytoplasmic incompatibility (CI). CI results in abortive embryonic development when infected males mate either with uninfected females or with females infected by incompatible *Wolbachia* variants. In a mixed population with infected and uninfected hosts, infected females have a reproductive advantage in facilitating the spread of *Wolbachia* (28). When different incompatible *Wolbachia* variants are present in a population, no stable coexistence can be theoretically maintained, resulting in a sweep until all coexisting variants are compatible (27) or in fixation of superinfection (12). Thus, *Wolbachia* represents a promising drive system for transgenes, reducing insects' ability to transmit pathogens (34).

CI results from inappropriate interactions between sperm and egg, leading to embryonic mortality in diploid species and to an excess of male production in haplodiploid species (reviewed in reference 38). CI is now considered to result from two bacterial components: a *mod* (for modification) function that affects sperm (*Wolbachia* strains are absent from mature sperm) and induces embryo death and a *resc* (for rescue) function provided by the *Wolbachia* strains present in the egg that restore compatibility (3, 21, 39). CI embryos from mosquitoes, flies, and wasps exhibit the same cytologic defects, suggesting a conservative mechanism induced by *Wolbachia* (38).

Among the host species studied so far, mosquitoes of the *Culex pipiens* complex exhibit the largest variability of CI crossing types with frequent uni- or bidirectional incompatible crosses (7, 14, 18, 20, 23). Such complexity is essentially driven by the high genetic diversity of *w*Pip variants (i.e., *Wolbachia* variants infecting *C. pipiens*), since more than 60 such variants have been identified (8) and since nuclear effects have never been observed on CI expression (1, 7, 13, 15, 18) except in a single case (33). *w*Pip genetic variability mostly affects mobile genetic elements (transposable element and WO prophage) but does not strictly correlate with the CI pattern, as shown by our analysis of 14 mosquito strains with 15 distinct polymorphic markers (7). However, we noticed a partial correlation between the *resc* function driven by females and WO prophage WD0580 (*Gp15*) gene variants.

Despite intense works on numerous species, the molecular basis of CI remains unsolved. Recently, published genome sequences revealed that *Wolbachia* variants infecting arthropods are unusual in that they contain a high number of genes encoding proteins containing ankyrin (ANK) repeats compared to the number found in mutualistic *Wolbachia* variants that infect filarial nematodes or in related α-proteobacteria (11, 29, 41). ANK repeats are ~33-residue sequence motifs devoid of enzymatic activity which mediates specific protein-

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protein interactions. ANK repeats have been found in toxins and numerous proteins involved in cell signaling, cytoskeleton integrity, intracellular trafficking, gene transcription, and cell cycle regulation (22, 32). Because of their ability to promote protein-protein interaction, ANK proteins might play pivotal roles in the establishment of *Wolbachia*-host relationships. A recent comparison of ANK genes of *Wolbachia* variants infecting the *Drosophila* genus showed significant differences between CI-inducing and non-CI-inducing variants, highlighting 10 candidate genes associated with reproductive parasitism in *w*Mel variants (i.e., *Wolbachia* variants infecting *Drosophila melanogaster*) (16). In the same line, Sinkins et al. (33) compared 18 ANK gene sequences of two bidirectionally incompatible *w*Pip variants and found two polymorphic WO prophage genes containing ANK domains, *pk1* and *pk2*, the latter being homologous to one of the 10 candidate genes found in *w*Mel. Furthermore, the host sex-specific expression of a *pk2* variant suggested that it might be involved in CI (33).

We challenged the hypothesis that ANK genes could be implicated in *C. pipiens* CI by examining the variability of 58 ANK genes in 14 genetically distinct *Wolbachia* variants which induce uni- and bidirectional CI. Differential expression of three polymorphic ANK loci was also examined to evaluate their implication in CI.

MATERIALS AND METHODS

Mosquito collections, *Wolbachia* variants, and crossing experiments. Fifteen laboratory strains of *C. pipiens* complex mosquitoes were used. For each strain, the reference, year, and country of origin are indicated in Table S1 in the supplemental material. Strains were reared in 65-cm^3 screen cages kept in a single room at 22 to 25°C, under a 12-h light/12-h dark cycle. Larvae were fed with a mixture of shrimp powder and rabbit pellets. Adult mosquitoes were fed with honey solution.

All strains except Ko and Tn were naturally infected by distinct *Wolbachia* variants (for more details, see reference 7). *Wolbachia* variants were discriminated by the transposable element *Tr1* marker (similar to the IS*5* *w*Mel element [6]) and by the presence or absence pattern of at least 10 WO prophage genes dispersed in the *w*Pip genome (8). Mosquito strains infected by different *Wolbachia* variants were subcloned to generate substrains, each infected by a unique *w*Pip variant. Fifteen mosquito strains harboring 14 distinct *Wolbachia* variants were studied. The uninfected strain SlabTC was created artificially by antibiotic treatment (7, 9) and was used as a control.

For each crossing experiment, 20 to 50 females and an equivalent number of males were mated. Two- to 5-day-old adults were used. Females were allowed to feed on blood 6 to 8 days after mating. Egg rafts were collected daily, and the CI status was estimated by the egg-hatch rate (HR), which was quantified by counting under binocular microscopy. If an egg raft produced no larvae, embryo development was checked to control insemination (for details, see reference 10). Egg rafts from noninseminated females were discarded. Incompatible crosses were repeated at least twice, and the results were pooled for analysis.

Screening for ankyrin domains. Contig DNA sequences for *w*Pip were obtained from the Wellcome Trust-Sanger Institute web site (http://www.sanger.ac.uk/Projects/W_pipientis/). Open reading frame (ORF) sequences larger than 30 amino acids were generated (in the six ORFs and without start codons) using the getorf program from the EMBOSS package (25). A total of 18,853 ORFs of 33 to 3,904 amino acids were obtained. ANK proteins were scanned for the presence of the ankyrin repeat region profile (PS50297; PROSITE database) using a generalized sequence profile method (pfsearch program from the PFTOOLS package [4]). Ankyrin repeat regions from 58 ORFs were detected with a score above the default threshold and were considered true positive. We used SMART, version 3.5, for graphical representation of ANK domains (see Fig. S1 in the supplemental material) (http://smart.embl-heidelberg.de/ [19, 31]).

PCR and sequencing of ANK domain genes. For each ORF, primers were designed to specifically amplify the ANK region (see Table S2 in the supplemental material). DNA was extracted using a CTAB protocol (26). For each ANK region, PCR was run for 30 cycles (94°C for 30 s, 52°C for 30 s, and 72°C for 1 min). PCR products were sequenced directly using the BigDye terminator kit and analyzed on an ABI Prism 310 sequencer. DNA sequences were aligned using CLUSTAL W software (37). As expected, all PCRs on the tetracycline-treated, *Wolbachia*-free strain (SlabTC) were negative, which confirmed the bacterial origin of these ANKs.

RT-PCR and real-time quantitative PCR. For each strain, three pools of five males and five females (adults just emerged) were analyzed. Mosquito total RNA was extracted from each pool using NucleoSpin RNA II kits (Macherey-Nagel) according to the manufacturer's protocol with the following modifications. RNA was digested for 30 min using 30 additional DNase I units to avoid genomic DNA contamination. First-strand cDNA synthesis was performed on 100 ng of total RNA using SuperScript II reverse transcriptase (RT) (Invitrogen) and 85 pmol of 10-nucleotide random primers. cDNA was purified on QIAquick minicolumns (QIAGEN) and eluted in 50 μl of water. For RT-quantitative PCR, each sample was analyzed in triplicate for ANK (*pk1*, *pk2*, and *ank2*), *wsp* (*Wolbachia* surface protein), and G6PDH (glucose-6-phosphate dehydrogenase) gene expressions. One microliter of cDNA was mixed with primers, 0.5 μM each (see Table S2 in the supplemental material), and 2 μl of anti-*Taq*-containing master mix and completed to 20 μl with water (master mix and anti-*Taq* antibody were used according to Roche LightCycler instructions for SYBR technology [40]). PCR was run for 45 cycles (94°C for 0 s, 60°C for 10 s, and 72°C for 15 s). The absence of genomic DNA contamination was checked by negative *wsp* amplification when RT was omitted. cDNA from SlabTC was used as control of expression.

Statistical analysis. ANK gene expression data were analyzed using generalized linear models. We analyzed, in seven strains (Lv, Bf-B, Ke-A, Ke-B, Au, Sl, and Mc), the differential expression of *Wolbachia* ANKs (*pk1*, *pk2*, and *ank2*) and *wsp*, corrected by nuclear gene expression (G6PDH gene) and by level of infection (*wsp*). Each mosquito sample was described by nine variables: strain (qualitative variable STR, seven levels), sex (qualitative variable SEX, two levels), *pk1*, *pk2*, *ank2*, and *wsp* expressions corrected for G6PDH gene expression (quantitative variables $pk1_M$, $pk2_M$, $ank2_M$, and wsp_M, respectively), and *pk1*, *pk2*, and *ank2* expressions corrected for *wsp* expression (quantitative variables $pk1_W$, $pk2_W$, and $ank2_W$, respectively). For all expression variables $pk1_M$, $pk2_M$, $ank2_M$, wsp_M, $pk1_W$, $pk2_W$, and $ank2_W$, the linear model SEX * STR (where "*" indicates additive and interactive effects between variables) was fitted to the data. This model was then simplified according to the method of Crawley (5) to allow the statistical grouping of strains displaying the same gene expression pattern. Calculations were performed using the R free software (24). Data were log-normal transformed for Gaussian analysis. Normality of residuals from the minimal model was tested using a Shapiro-Wilk test (JMP module; SAS Software).

Nucleotide sequence accession numbers. Partial sequences of ANK gene variants of *pk1*, *pk2*, *ank2*, and *ank12* were submitted to the EMBL database under the following accession numbers: AM397068 to AM397079 and AM503576 and AM503577.

RESULTS

Crossing relationships. We extended a previous CI analysis (7) by including Ep-A and Ep-B strains, which represent here a total of 225 crosses. For each cross, HRs were quantified and classified as compatible (HR $\geq$ 70%), intermediate (70% $>$ HR $\geq$ 30%), or incompatible (HR $<$ 30%), according to the bimodal HR distribution classically observed in *C. pipiens* (17, 14, 7). We never observed incompatibility in intrastrain crosses or hatching heterogeneity (i.e., the production of both compatible and incompatible egg rafts from a single cross), indicating that each strain contains a unique crossing type (see Table S3 in the supplemental material). For interstrain crosses (n = 210), we obtained 168 compatible (80%) and 42 incompatible (20%) ones. Twenty-two strain combinations displayed unidirectional CI, while 10 displayed bidirectional CI, all involving the Istanbul strain that is a high CI inducer. Each strain was described by its cytotype, i.e., its pattern of cytoplasmic crossing types with all other strains. This led to the identification of 14 distinct cytotypes among the 15 mosquito strains (Ka-C and Ma-A behaved identically in all crosses). To evaluate the similarities of *resc* and *mod* abilities between strains, we considered the patterns of crossing types of females and males independently. For each strain, females and males were

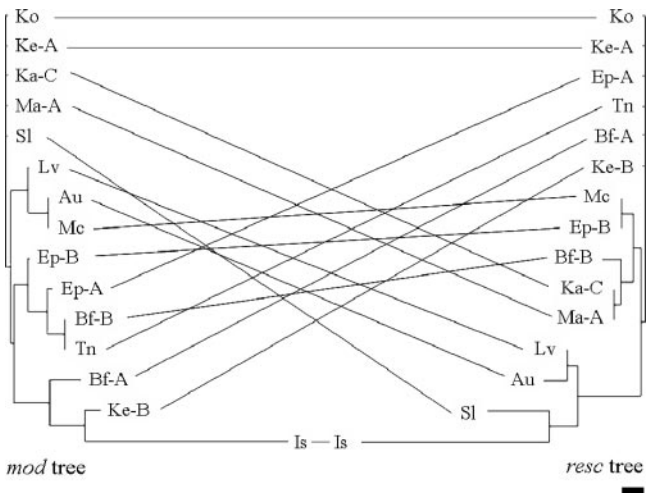

FIG. 1. Distance tree comparison of *mod* and *resc* phenotypes of 15 mosquito strains. Branch lengths were proportional to the amount of cross changes. Bar, one cross change.

each described by a 15-bit digit, with each bit representing the compatibility status with other strains; for the order, see Table S3 in the supplemental material. Clustering, built by the neighbor-joining method (Fig. 1), identified eight distinct groups for the females, i.e., the *resc* ability, and nine for the males, i.e., the *mod* ability. Interestingly, female and male clusterings were different, which indicates that the *mod* and *resc* functions are probably driven by independent genetic entities.

Polymorphism of ANK domains in *w*Pip. A *w*Pip genome scan for ankyrin repeats using the PROSITE PS50297 profile identified 60 unique ANK regions encoded by 58 distinct ORFs (two ORFs each encoded two distinct ANK regions) (see Table S2 in the supplemental material). This probably represents the whole ANK gene family, although the final score must await the completion of the *w*Pip genome assembly, which is still in progress. As a control, scanning the fly *wMel* genome with this profile identified the same 23 proteins previously identified by Wu et al. (41). *w*Pip and *w*Mel share only nine highly similar ANK genes (50 to 85% identity at the amino acid level) and five moderately similar ones (40 to 45% identity) (not shown). As a first screen for polymorphism, we used Ko, Tn, Is, and Sl strains, which display distinct CI patterns. All ANK ORFs, except *ank40*, were detected in every strain; *ank40* was not amplified from Is and Sl DNA even using five additional primer sets for the PCR (Table 1). For each mosquito, ANK products were all monomorphic, in agreement with the absence of multiple infections in *C. pipiens* (8). Fifty-one ANK sequences, including *ank40* sequences of all positive strains, were strictly identical at the nucleotide level to those found in the *w*Pip genome (Pel strain). Four ORFs (*pk1*, *pk2*, *ank2*, and *ank12*) showed nonsilent polymorphisms within ANK regions (Table 1 and see Fig. S1 in the supplemental material) and three (*ank4*, *ank5*, and *ank6*) showed nonsilent polymorphisms outside the regions (data not shown).

We next extended the analysis of *pk1*, *pk2*, *ank2*, and *ank40* to the 15 strains. Although *pk2* polymorphism, like *ank12*, did not strictly correlate with CI (they each showed identical alleles in different CI subgroups; strains Ko and Tn versus strain Is or Sl [Table 1]), we did not exclude *pk2* in the study because of a recent report that describes it as polymorphic and potentially involved in CI (33). We identified five *pk1*, four *pk2*, and five *ank2* alleles. The products of the five *pk1* alleles showed 1.8 to 16.4% predicted amino acid divergence, the products of the four *pk2* alleles showed 2 to 7.4% divergence, and the products of the five *ank2* alleles showed deletions of 20 to 51 residues. PK1a (Ep-A, Bf-A, Ko, Tn, and Ep-B), PK1d (Is), and PK1e (Ka-C and Ma-A) predicted proteins contained 10 ANK repeats followed by two transmembrane regions, while substitutions degenerated the second ANK domain in PK1b (Mc, Bf-B, and Sl) and PK1c (Ke-A, Ke-B, Lv, and Au) (see Fig. S1 in the supplemental material). The four PK2 predicted proteins displayed organizations that were identical with three ANK domains. Concerning *ank2*, the five alleles encoded 99% similar predicted proteins with either four (ank2a [Ep-A, Bf-A, Ko, Tn, and Ep-B]), five (ank2d [Ka-C and Ma-A], ank2e [Ke-A, Ke-B, Lv, and Au]), or five and a half (ank2b [Mc, Bf-B, and Sl]; ank2c [Is]) ANK repeats, preceding two transmembrane regions. Last, further *ank40* analysis detected the same allele in six strains (Ep-A, Ep-B, Bf-A, Ko, Tn, and Au) and none in the remaining nine strains (Table 1), which strengthens the notion that *ank40* polymorphism is restricted to absence or presence.

We next asked whether ANK polymorphism might correlate with CI patterns. Increasing the sample size to 15 strains did not show up a correlation of *pk2* and *ank40* allelic distributions with CI patterns, as it was already the case during the first screen of Ko, Tn, Is, and Sl strains. *pk1* and *ank2*, which both correlated with the *resc* function in the first screen, appeared in full linkage desequilibrium in the 15 strains

TABLE 1. Alleles of variable ANK genes present in *Wolbachia* variants infecting 15 *Culex pipiens* strains[a]

Strain[b]	Allele for ANK gene				
	pk1	*pk2*	*ank2*	*ank12*	*ank40*
Ep-A	a	d	a		a
Bf-A	a	a	a		a
Ko	a	a	a	a	a
Tn	a	a	a	a	a
Ke-A	c	a	e		−
Ke-B	c	a	e		−
Mc	b	b	b		−
Ep-B	a	d	a		a
Bf-B	b	c	b		−
Ka-C	e	a	d		−
Ma-A	e	a	d		−
Lv	c	a	e		−
Au	c	a	e		a
Sl	b	b	b	a	−
Is	d	a	c	b	−

[a] Only five ANK genes presented polymorphisms in the four strains widely studied (Ko, Tn, Sl, and Is). For *pk1* and *pk2*, allele a corresponds to the Pel strain and allele b corresponds to the Bei strain (32). −, strains in which *ank40* was not detected and empty cases to untested strains.

[b] Strains in the same CI subgroup (behaving identically based on their *resc* ability) are grouped together.

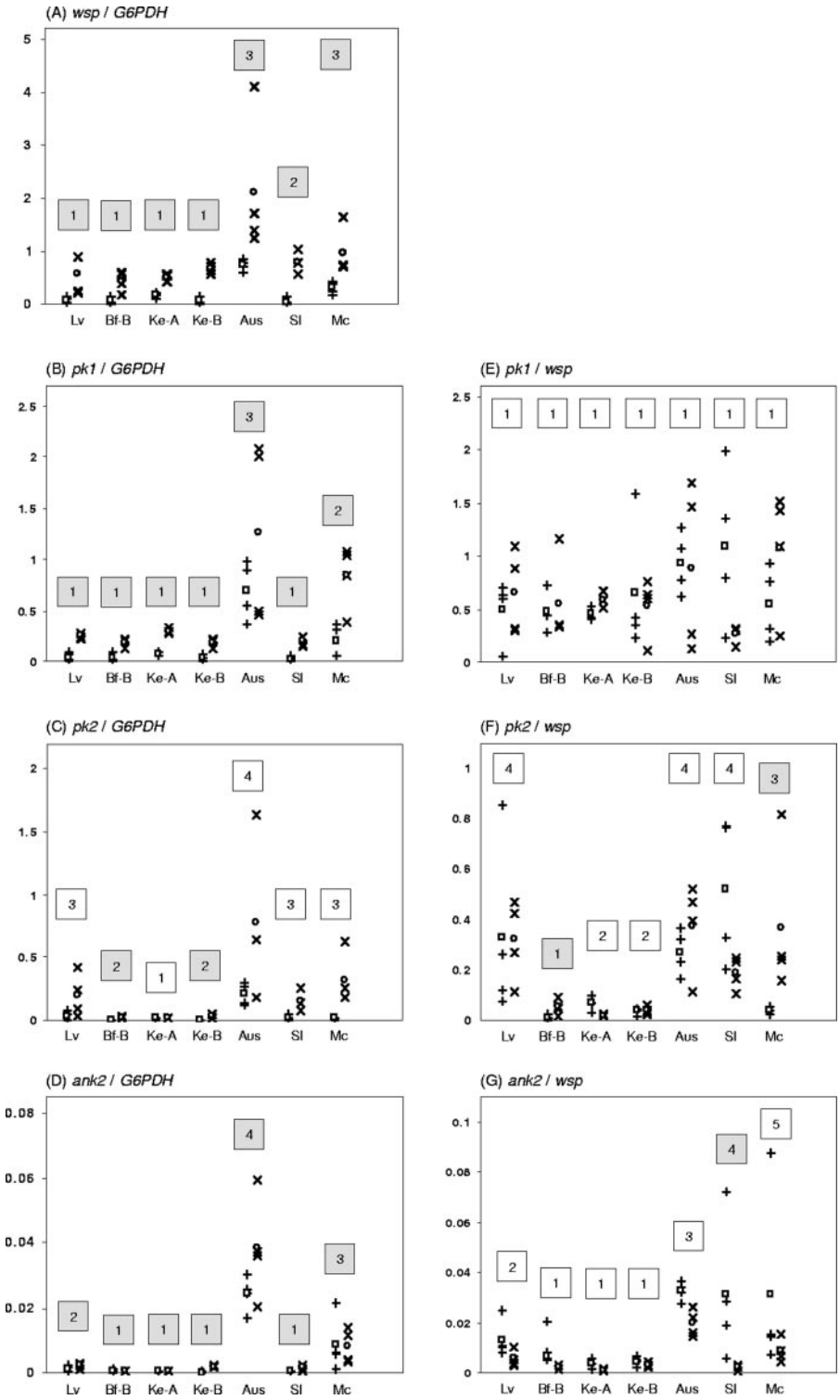

FIG. 2. Expressions of the *wsp* and ANK genes of *Wolbachia* variants of seven mosquito strains. Expressions of *wsp*, *pk1*, *pk2*, and *ank2* were provided relative to G6PDH gene expression (A, B, C, and D, respectively). Expressions of *pk1*, *pk2*, and *ank2* were provided relative to *wsp* expression (E, F, and G, respectively). Pluses, males; crosses, females; open squares, means of males; open circles, means of females. Boxed numbers refer to statistical group, and gray coloring indicates a significant difference between sexes.

(Table 1) and lost the correlation when the eight additional *resc* groups were included (the same alleles were found in strains with different *resc* patterns, e.g., in Mc and Sl, and conversely, distinct alleles were found in strains with identical *resc* patterns, e.g., in Mc and Ep-B). These results indicate that although probably inducing different functional outcomes, the polymorphism of ANK genes cannot explain the CI pattern of *C. pipiens.*

Variable expression of ANK genes. Variable ANK gene expression was previously described for *C. pipiens* mosquitoes (30, 33). This might modulate CI penetrance and should thus be taken into account for the correlation tests. We studied seven mosquito strains, describing two groups (Lv, Ke-A, Ke-B, and Aus and Bf-B, Sl, and Mc) that each shared identical *pk1*, *pk2*, or *ank2* alleles but differed in their cytotypes (Table 1 and Fig. 1; see Table S3 in the supplemental material). Strain- and sex-dependent expressions of *pk1*, *pk2*, and *ank2* genes were estimated by real-time quantitative PCR. *wsp* gene expression was used as a control to correct for *Wolbachia* infection level, and the G6PDH gene was used to correct for mosquito size and cDNA quality. *pk1*, *pk2*, and *ank2* were expressed in males and females of all strains, with strain- and sex-dependent variations after G6PDH gene normalization and with interactive effect for *pk2* (Fig. 2B, C, and D). *pk1* and *ank2* distributed into three and four statistical strain groups, respectively, and showed sex-dependent expression in all strains. *pk2* distributed into four statistical strain groups, of which only group two showed significant sex-dependent variations. In all sex-dependent cases, ANK gene expression was always higher in females (Fig. 2B, C, and D). Interestingly, *wsp* showed similar sex- and strain-dependent expression with interactive effect, suggesting that the level of infection accounts for a major part in the observed variations (Fig. 2A). Indeed, after *wsp* normalization, *pk1* expression became independent from sex and strain (Fig. 2E), while *pk2* and *ank2* expression still showed a strain effect (four and five statistical groups, respectively; Fig. 2F and G), with a much reduced sex effect for *ank2* (compare Fig. 2D and G). However, even after correcting with the G6PDH gene only, *pk1* and *ank2* did not show better correlations with the *resc* function. For example, Lv and Ke-A/Ke-B showed different CI patterns but identical *pk1* levels (Fig. 2B). Similar findings were observed for *ank2* in Bf-B and Sl (Fig. 2D). Conversely, Lv and Aus with closely related cytotypes showed differential expression levels (Fig. 2B and D). Taken together, our data show that the 14 cytotypes observed in the 15 strains do not correlate with ANK gene polymorphism, even taking into account strain- and sex-dependent gene expression.

DISCUSSION

How *Wolbachia* strains manipulate host reproduction is critical for the understanding of the biology and evolution of this bacteria. Among candidate genes, ANK genes have received focused attention as they appeared widespread in the genomes of *Wolbachia* strains inducing reproductive parasitism, whereas few are present from those of mutualistic lineages in filarial nematodes (11, 41, 29). Functionally, ANK domains have the capacity to interact with host proteins, and nonsilent ANK polymorphism was reported recently for *w*Mel and *w*Pip, leading to the hypothesis that ANK might be involved in CI determinism (16, 33). In the present study, we addressed the variability of all *wPip* ANK genes in 15 incompatible mosquito strains, from which we identified 14 distinct *Wolbachia* variants associated with 14 crossing types, in agreement with previous findings that CI occurs at a high frequency in *C. pipiens* (7, 14, 18, 20, 23). Strain clustering according to *mod* or *resc* similarity produced disjunctive groups (Fig. 1), supporting the notion that both functions are encoded by distinct genes (3, 21, 39).

We identified 60 ankyrin regions in 58 unique ORFs in the *w*Pip genome (Pel strain). Although the genome assembly is still in progress, the level of coverage makes it unlikely that additional ANK genes were missed. These 58 represent the highest number of ANK genes found in a *Wolbachia* genome. All ANK genes, except *ank40*, were found in the 15 strains analyzed; *ank40* was absent from 9 strains. The fact that PCR failed using six different amplimer sets in all *ank40*-negative strains strongly suggests that *ank40* is indeed absent and simply not divergent enough to be amplified. It is thus likely that additional ANK genes exist in other *w*Pip genomes, while being absent from Pel *w*Pip. Strain-to-strain difference in ANK repertoire has already been shown between *w*Mel and *w*Sim (one of the *Wolbachia* variants that infects *Drosophila simulans*), the latter coding for seven additional ANK genes (29). Besides, of the 60 ANK gene regions sequenced in four incompatible *w*Pip variants, only five were polymorphic; the others were strictly identical to those found in the Pel *w*Pip genome. Such variability is very low compared to that of the 10 polymorphic ANK genes (of 23) between *w*Mel and the closely related non CI-inducing *w*Au (16). Interestingly, aside from *ank40*, the other four polymorphic ANK loci were variable in both DNA and predicted amino acid sequences. In particular, *pk1* and *ank2* alleles encode peptides that differ by one ANK repeat or by the spacing between two repeats. This delineates two contrasting situations, one in which most ANK sequences are strictly conserved at the nucleotide level in the strains described here and in Pel, the other in which a few derived ANK peptides differ in their domain organizations. These situations might reflect phage origins, since the variable *pk1* and *pk2* have been shown to be located in WO prophage regions (33). This might also be true for *ank2*, in linkage disequilibrium with *pk1* (this study) and also with WD0580 (*Gp15*), a WO prophage marker that partially correlates with the *resc* function (7, 8; unpublished data). Final genome assembly will give a definite answer on the origins of *ank2*, *ank12*, and *ank40*.

The goal of this study was to examine whether and which variable ANK genes might code for CI determinants. To this aim, we compared strain distributions of all variable ANK alleles with crossing types. Assuming that different alleles should at least be found in incompatible strains, our analysis clearly rejects the direct implication of any of the ANK genes identified. This conclusion does not support the proposed role of *pk2*, deduced from the analysis of only four strains (33). In the report of Sinkins et al. (33), *pk2* was also shown to be expressed only in females of some strains. Host- and sex-specific expression could thus represent a confounding factor that affects CI penetrance, and we considered it in the correlation test. In our study, *pk1*, *pk2*, and *ank2* were found expressed in all strains and in both sexes but at different levels (Fig. 2). Since similar variations were also detected for *wsp* after G6PDH gene normalization, this result strongly suggests that bacterial density is probably a major variable factor. However, even after *wsp* normalization, strain effects and, to a lesser extent, a sex effect were still maintained for *pk2* and *ank2* (Fig. 2F and G), whereas *pk1* behaved identically to *wsp* in all strains and in both sexes. In particular, Sl males showed higher *ank2* expression than did Sl females, whereas Mc and Bf-B males

expressed less *pk2* than did Mc and Bf-B females. Using either *wsp* or G6PDH gene normalization, the observed CI pattern did not correlate with the strain- or sex-dependent expression of any ANK gene. This excludes their direct implication as CI determinants, although they might well play a role in events downstream of the initial trigger. Alternatively, ANK genes might be involved in other molecular cross talks with the host, irrelevant to CI. For instance, *Wolbachia* density increased in insecticide-resistant *C. pipiens* mosquitoes without noticeable effect on incompatibility (2, 9). Variable expression of ANK genes might thus just reflect a metabolic response, such as those involved in the active import of essential of amino acids, carbohydrates, or lipids from the host cell, which *Wolbachia* strains are unable to produce, as shown for *w*Mel (41).

Our identification here of 14 distinct crossing types among the 15 *C. pipiens* strains illustrates the complexity of CI in this species. The lack of transitivity in the crossing relationships combined with the occurrence of both uni- and bidirectional incompatibility favors a multifactorial determinism. In particular, our data support the notion that CI is driven by at least two distinct genetic units that control the *mod* and *resc* functions independently. However, each function is probably determined or at least modulated by several factors, since we evidenced nine *mod* and eight *resc* groups among the 15 strains. This is supported by the observation that lethal embryos issued from parents infected by incompatible *Wolbachia* variants develop further than when only males are infected, suggesting that specific CI determinism could be invoked in CI crosses according to mother infection (10). CI complexity in *Culex* thus makes the identification of determinants by formal genetics elusive, all the more so that penetrance appears variable depending on which pair of infected strains are studied (10). Postgenomic tools remain to be set up to address more directly which host functions are differentially affected by incompatible *Wolbachia* variants and which bacterial components are responsible for it.

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