On the mechanism of *Wolbachia*-induced cytoplasmic incompatibility: confronting the models with the facts

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Summary

The endocellular bacterium *Wolbachia* manipulates the reproduction of its arthropod hosts for its own benefit by various means, the most widespread being cytoplasmic incompatibility (CI). To date, the molecular mechanism involved in CI has not been elucidated. We examine here three different CI models described in previous literature, namely, the ''lock-and-key'', ''titration–restitution'' and ''slow-motion'' models. We confront them with the full range of CI patterns discovered so far, including the most complex ones such as multiple infections, asymmetrical and partial compatibility relationships and the existence of *Wolbachia* variants that can rescue the host from CI but not induce it. We conclude that the lock-and-key model is the most parsimonious of the models and fits the observations best. The two other models cannot be categorically invalidated, but they encounter some difficulties that make additional hypotheses necessary. *BioEssays* 25:259–265, 2003. © 2003 Wiley Periodicals, Inc.

Introduction

Cytoplasmic incompatibility (CI) is a reproductive incompatibility observed in many arthropod species, which is caused by the endocellular bacterium *Wolbachia* (α-proteobacteria: Rickettsiaceae) (reviewed in Refs. 1,2). In its simplest form, CI can be described as an embryonic mortality that occurs when uninfected females mate with *Wolbachia*-infected males. Infected females are fully fertile regardless of the infection status of the male. As a consequence, infected females have more offspring on average. This allows the maternally inherited bacterium to invade new host populations.

The means by which *Wolbachia* induce CI are currently unknown, however, there is a general consensus that *Wolbachia* must somehow modify the sperm, since embryonic development aborts when sperm from an infected male fertilize an uninfected egg, due to the loss of improperly condensed paternal chromosomes (a notable exception is found in haplo-diploid arthropods species, where the loss of paternal chromosomes is not lethal but leads to haploid embryos that develop normally as males). This modification of the sperm must take place at an early stage of spermatogenesis because the bacteria are shed from maturing sperm and eliminated in cytoplasmic ''waste-bags''.[3] It is also known that such modified sperm will be fully functional if *Wolbachia* are present in the egg, which implies that some sort of ''rescue'' is performed by those *Wolbachia*. These ideas were formalized by Werren,[4] through the ''*mod resc*'' (modification/rescue) model, which involves two functions: *mod* modifies sperm while *resc* takes place in the egg and restores paternal material functionality. This *mod resc* (or poison antidote) model is a useful general concept: there is no restriction with regard to the actual nature of the *mod* and *resc* functions. Three biochemical models have been proposed so far to translate *mod* and *resc* into more concrete factors: the ''lock-and-key'' model, the ''titration–restitution'' model and the ''slow-motion'' model. The aim of the present article is to test these different propositions by confronting them with several key observations from studies of CI.

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The models

1. The ''lock-and-key'' hypothesis (Fig. 1)

Following this model, the *mod* function is due to the production by the bacteria of a ''lock'' that binds to component of the paternal nucleus. Embryonic mortality occurs in crosses between infected males and uninfected females because the paternal material is ''locked-in'' and therefore unable to perform correctly. On the contrary, eggs infected by *Wolbachia* remain compatible with such modified sperm because the bacteria present in the egg produce a ''key'' that removes the lock (*resc* function). The two important features of this proposition are that (i) *mod* and *resc* do not result from the same molecular mechanism and are determined by different bacterial genes and (ii) *mod* penetrates the egg together

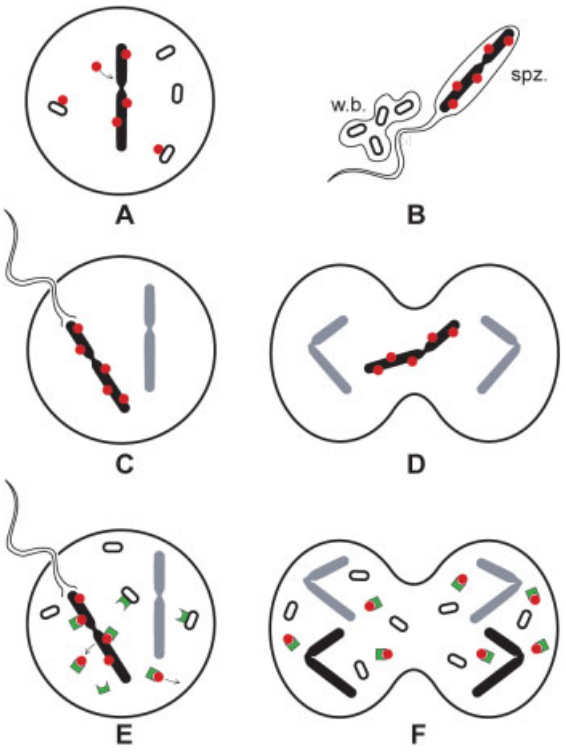

Figure 1. Lock-and-key model.**A,B:** Spermatogenesis in an infected male. **A:** *Wolbachia* (white symbols) produce a lock, (red circle) binding for example to paternal chromosomes (large black bar). **B:** The bacteria are then shed in a waste-bag structure (w.b.) with most of the cytoplasm, and are therefore absent from the mature spermatozoon (spz). **C, D:** Incompatible cross between an infected male and an uninfected female. **C:** The sperm cell transporting "locked" paternal chromosomes enters an uninfected egg and meets unmodified maternal chromosomes (grey bar). **D:** In the absence of a key to remove the lock, paternal chromosomes are not functional and only maternal chromosomes take part normally in mitosis, which results in CI (death of the embryo in diplo-diploid species, production of a haploid male offspring in some haplo-diploid species). **E,F:** Compatible cross between two infected individuals. **E:** In an infected oocyte, *Wolbachia* produce a key (green symbol). **F:** The lock is thus removed from paternal chromosomes and mitosis takes place normally, rescuing the embryo.

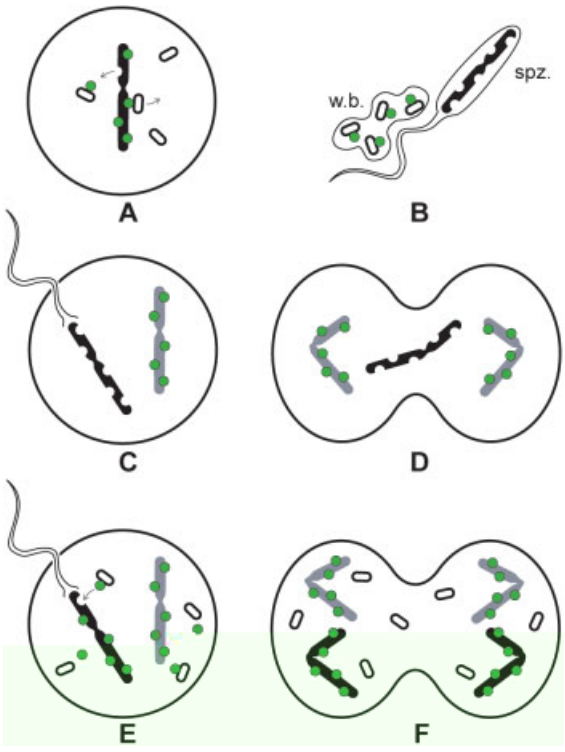

Figure 2. Titration–restitution (= sink) model. **A,B:** Spermatogenesis in an infected male. **A:** *Wolbachia* (white symbols) titrate-out a host protein (green circles) which is normally associated with chromosomes. **B:** The titrated protein is then expelled from the cell when *Wolbachia* are shed from the maturing spermatocyte, with most of the cytoplasm, in a waste-bag structure (w.b.). Paternal chromosomes (black bar) in the mature spermatozoon (spz) are therefore missing the protein and are not functional. (NB: In an infected oocyte, the same phenomenon applies but the titrated-out protein is not lost and remains temporarily associated with *Wolbachia* in the cytoplasm of the oocyte). **C,D:** Incompatible cross between an infected male and an uninfected female. C: The "titrated-out" sperm cell enters an uninfected egg: due to lack of the host protein, paternal chromosomes are not functional and only maternal chromosomes (grey bar) take part normally in mitosis (D), which results in CI. **E,F:** Compatible cross between two infected individuals. **E:** In an infected oocyte, the *Wolbachia* give back to maternal and paternal chromosomes the host protein previously titrated-out. **F:** mitosis can now proceed normally, which rescues the embryo.

with paternal chromosomes, allowing a direct physical interaction between *mod* and *resc* products. This model has been proposed in several papers,[4–7] but molecular evidence for it is lacking so far.

2. The "sink" or "titration–restitution" hypothesis (Fig. 2)

Kose and Karr[8] selected monoclonal antibodies raised against partially purified *Wolbachia* extracts. They first observed that, as expected, the anti-*Wolbachia* antibodies associated strongly with *Wolbachia* in infected *Drosophila simulans* eggs, and did not associate at all with condensed chromosomal DNA from the host. Yet, unexpectedly, the same antibodies produced a faint but reproducible signal in association with condensed chromosomes of the host if the egg was uninfected. Such a signal has also been observed on host DNA in *Drosophila simulans* spermatocytes (C. Lassy, H. Kose and

T.L. Karr unpublished results quoted in Ref. 8). Furthermore, anti-*Wolbachia* antibodies appeared to target histone-like host proteins.

Such observations led the authors to suggest that CI (that is, the *mod* function) might be due to *Wolbachia* removing some proteins normally associated with host chromosomes[8,9] as previously suggested by Werren.[4] Such titration would also occur in infected eggs prior to fertilization, as suggested by the absence of signal on host chromosomes in infected eggs. Presumably, the *Wolbachia* would give back the proteins to all chromosomes after fertilization (*resc* function). Under this view, *mod* and *resc* might be determined by the same gene(s) (the shift from titration to restitution after fertilization would then be triggered by host regulatory factors) or by different genes: one encoding a titrating factor, and the second encoding an inhibitor of titration, resulting in restitution.

3. The "slow-motion" hypothesis (Fig. 3)

Callaini et al.[10] observed that, in incompatible *Drosophila* embryos, paternal chromosomes can condense and produce an anaphase-like aspect during the first mitosis, albeit *later* than maternal chromosomes do, suggesting that *mod* is merely delaying—and not completely blocking—the entry into mitosis. More recently,[11] Tram and Sullivan extended this observation to hymenopterans, and further showed that nuclear envelope breakdown, which marks the entry into mitosis, is also delayed for the paternal material.

These workers (see also Ref. 12) thus postulated that CI is due to *Wolbachia* altering the timing of the first mitosis and more specifically, that (i) *mod* is due to *Wolbachia* producing a factor that first binds to paternal chromosomes and then slows down their movements during the first embryonic mitosis, leading to unsynchronized paternal and maternal sets, and (ii) *resc* is caused by the similar modification of maternal chromosomes when *Wolbachia* are present in the egg, restoring a synchronous cycle between paternal and maternal complements.

The originality of this model is that the *resc* function is not caused by the removal of the slowing-down factor (otherwise we would be back to a subtype of the lock-and-key model) but to the production of the same factor in the egg. In other words, *mod* and *resc* result here from the same molecular mechanism and are determined by the same bacterial gene(s). Modified in the same fashion, the paternal and maternal set would be synchronized during the first mitosis. Although the delayed entry into mitosis of paternal material is now well supported, the "rescue" aspect of this model is still speculative: it remains to be demonstrated that maternal chromosomes movements are indeed slowed down when the egg is infected. In the present paper, when mentioning the "slow-motion" model, we will refer more particularly to the hypothesis that *mod* and *resc* indeed constitute a single slowing down factor.

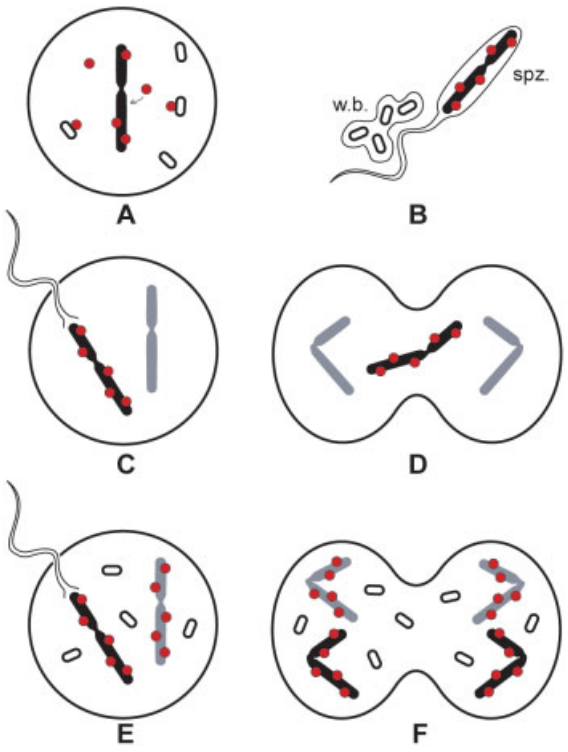

Figure 3. Slow-motion model. **A,B:** Spermatogenesis in an infected male. **A:** *Wolbachia* (white symbols) produce a slowing down factor (red circles) binding for example to paternal chromosomes (black bar) in such a way that they will go through the initial stages of mitosis at an abnormally slow pace. **B:** The bacteria are shed from the maturing spermatocyte, with most of the cytoplasm, in a waste-bag structure (w.b.). Paternal chromosomes in the mature spermatozoon (spz) remain loaded with the slowing down factor. (NB: in an infected oocyte, *Wolbachia* modify maternal chromosomes in the same way). **C,D:** Incompatible cross between an infected male and an uninfected female. **C:** a sperm cell bearing "slowed-down" paternal chromosomes enters an uninfected egg. **D:** by lagging behind maternal chromosomes (grey bar) during mitosis, paternal chromosomes are partially or totally lost, which results in CI. **E,F:** Compatible cross between two infected individuals. **E:** The sperm cell bearing "slowed-down" paternal chromosomes enters an oocyte infected by *Wolbachia*. **F:** Since maternal chromosomes are slowed down in the same fashion, both chromosome sets are synchronous, and the first mitosis proceeds normally (albeit at a slower pace than usual).

Confronting the models with the facts

In this section, we present several important features of CI, each representing a test for the models described above. The basics of CI are as follows (i) when an infected male mates with an uninfected female, embryonic mortality occurs, but (ii) the very same male will be fully fertile if the female is infected by the same *Wolbachia*. Since the three CI models have been

designed first to account for those two basic observations, they can all explain them in a satisfactory manner. Accordingly we will not discuss those two features again. Yet, CI presents several other characteristics, which are not so easily explained by all models.

A. The resc function does not interfere with normal sperm cells

Eggs infected by one or several *Wolbachia* strains are fully compatible with sperm from uninfected males. No exception to this rule has been found so far.

1. *Lock-and-key hypothesis.* The "key" molecule present in the infected egg will not interfere with normal paternal material, since the key interacts only with its specific lock molecule.

2. *Titration–restitution model.* Implicitly, it is assumed that a normal paternal chromosomal set (carrying its usual load of associated proteins) will not be affected by *Wolbachia* making available more of these molecules upon its entry into the egg. We see no particular reason either to dismiss or to confirm this hypothesis.

3. *Slow-motion hypothesis.* Callaini et al.[10] are aware that their model, without any additional hypothesis, would predict incompatibility between a normal sperm cell (paternal chromosomes with normal kinetics) and an infected egg (maternal chromosomes slowed-down by the bacterial factor). They postulate, therefore, that the amount of "slowing down factor" is sufficient in an infected egg to synchronize incoming normal chromosomes in step with the maternal set, when they write:(10) "*Presumably, the male chromatin recruits the Wolbachia-derived factor from the oocyte cytoplasm during replication of DNA. Maternal and paternal chromatin condensation are therefore coupled and the first mitotic division takes place successfully*". It remains to be established whether the first mitosis is systematically slower when the female is infected, as predicted by this model.

B. mod and resc interact in a specific manner

CI occurs when infected males mate with uninfected females. However, embryonic mortality is also observed when the two partners bear different *Wolbachia* strains.[13] In such cases, CI occurs in both directions of cross, and is thus termed bi-directional (as opposed to uni-directional CI, occurring in crosses involving only one *Wolbachia* strain). Bi-directional CI demonstrates that *mod* and *resc* interact in a specific manner: a *Wolbachia* strain is only compatible with itself (some exceptions to this general rule are discussed below, see point C).

1. *Lock-and-key hypothesis.* Allowance for bi-directional incompatibility is built into the basics of the lock-and-key model. Indeed, one can envision the existence of a virtual infinity of possible lock/key combinations.

2. *Titration–restitution hypothesis.* A first hypothesis to explain bi-directional incompatibility between different *Wolbachia* variants would be that each variant titrates-out and restitutes a different DNA-binding host protein. Such an explanation might not allow for a very wide diversity of compatibility types. A second, more flexible, option would be that each *Wolbachia* has a specific "titration–restitution profile" among the proteins available on the host chromosomes. A small number of target molecules might then allow a larger number of different compatibility types.

3. *Slow-motion hypothesis.* *This model would explain bi-directional incompatibility because paternal and maternal chromosomes affected by different slowing-down factors would be asynchronous. Two different hypotheses must be distinguished here. First, different slowing down factors might bind to the same sites on host chromosomes. Maternal and paternal factors would then compete to bind on paternal chromosomes. In some types of crosses, this would lead to maternal chromosomes being more delayed than paternal ones, which should result in the loss of maternal chromosomes. Yet, experiments using eye mutation markers in the hymenopteran genus Nasonia have shown that it is always the paternal set that is lost in both directions of cross.[5] This hypothesis is thus unlikely, although this observation needs to be generalized to other Wolbachia/host associations. An alternative view is that the slowing down factors produced by different Wolbachia bind to different sites. Paternal chromosomes, already slowed down by the paternal factor, would be slowed down further by the factor of maternal origin upon its entry into the egg. Empirical observations would then fit the model's prediction: the loss of paternal chromosomes in all incompatible crosses. Thus, the slow-motion model requires that bi-directionally incompatible Wolbachia strains produce factors binding to different chromosomal sites, so that the effect of the slowing-down factor present in the egg cytoplasm can add to the effect of the factor produced in sperm.*

C. Two Wolbachia variants can be partially and asymmetrically compatible

D. simulans females artificially transinfected by the *Wolbachia* *w*Mel, normally found in *D. melanogaster*, are partially capable of rescuing sperm of males infected with the *Wolbachia* *w*Ri, naturally infecting *D. simulans* (i.e. only 25–30% of the embryos die) although males infected by *w*Ri induce a nearly total embryo mortality (95–100%) when mated with uninfected females. In the reverse cross, *w*Ri-infected females fully rescue sperm from males infected with *w*Mel, although *w*Mel-infected males induce total embryo mortality when mated with uninfected females.[14] Thus, the *resc* function of *w*Ri is fully

efficient against the *mod* function of *w*Ri, which is trivial, but also against the *mod* function of *w*Mel, which is quite unexpected, since the two variants are clearly distinct, based on two independent molecular markers.[15,16] Likewise, one can conclude from partial compatibility in the reverse cross that the *resc* function of *w*Mel is partially capable of rescuing embryos when faced with the *mod* function of *w*Ri.

1. *Lock-and-key hypothesis.* To explain the above pattern, one could postulate that the locks of *w*Mel and *w*Ri are relatively similar. *w*Ri would have a wider-spectrum key, allowing it to ''open'' both the *w*Ri and *w*Mel locks. On the other hand, *w*Mel would have a more specific key, which would not be very good at opening the *w*Ri lock, explaining the imperfect rescue in the other cross.

2. *Titration–restitution hypothesis.* The situation of *w*Ri and *w*Mel can be explained if the two variants remove and restitute the same host molecule, with *w*Ri showing a higher affinity for it than *w*Mel. In crosses between *w*Ri males and *w*Mel females, *Wolbachia* in the egg would not restitute enough host molecule to paternal chromosomes, resulting in partial embryonic mortality. On the contrary, in crosses between *w*Mel males and *w*Ri females, *Wolbachia* would restitute even more host molecule than necessary to paternal chromosomes, resulting in full rescue.

3. *Slow-motion hypothesis.* In this case, the *w*Ri/*w*Mel relationship can be explained if the slowing down factors produced by the two variants bind to the same sites on host chromosomes, with *w*Ri showing a higher affinity for those sites than *w*Mel. In crosses between *w*Ri males and *w*Mel females, *Wolbachia* in the egg would not be able to make maternal chromosomes as slow as paternal chromosomes resulting in partial embryonic mortality. On the contrary, in crosses between *w*Mel males and *w*Ri females, paternal chromosomes would be further slowed down by the maternal factor, resulting in full rescue.

D. Different mod functions do not exclude one another

Sperm from males infected simultaneously by two different CI-inducing *Wolbachia* will induce embryonic mortality if the eggs bear only one of the two *Wolbachia* variants.[17–20] Moreover, cases of triple infections lead to similar conclusions: embryonic mortality occurs if females do not bear all the *Wolbachia* variants present in males.[21] Therefore, a single sperm cell can bear the mark of two or three different *mod* functions simultaneously.

1. *Lock-and-key hypothesis.* Paternal nuclei bearing two different locks will remain impaired unless the *two* corresponding keys are present in the egg.

2. *Titration–restitution hypothesis.* If each *Wolbachia* titrates-out and gives back a different (or a different spectrum of) DNA-binding protein(s), two different *Wolbachia* variants acting together in the same maturing sperm will lead to a new and unique pattern of titration for paternal DNA. Accordingly, this DNA will not be rescued in an egg bearing only one of the two *Wolbachia* variants.

3. *Slow-motion hypothesis.* If different slowing down factors act additively, that is, if they bind to different sites, paternal DNA affected by two *Wolbachia* will be more severely slowed-down than that modified by one of the two *Wolbachia* in the egg, leading to a failed mitosis.

E. Different resc functions do not exclude one another

Eggs infected simultaneously by two different and incompatible *Wolbachia* strains (say A and B) will not suffer from CI when fertilized by sperm cells from males infected by A, by B, or by A and B.[17–20] This pattern holds true in the case of triple infections: females infected simultaneously by three *Wolbachia* are compatible with any male infected by one, two or three of these bacteria.[21]

1. *Lock-and-key hypothesis.* The *resc* function is due to the direct physical interaction between a key and its specific lock. There is, therefore, no reason why two different keys should exclude one another. If keys A and B are present simultaneously in the egg, any paternal nucleus locked by locks A, B or A and B will be rescued.

2. *Titration–restitution hypothesis.* If two or more *Wolbachia* differ in the (spectrum of) molecule(s) they titrate-out in spermatocytes and give back in the egg, *resc* functions are additive: in a bi-infected egg, each variant will give back two different sets of proteins and then restore compatibility with sperm missing partially (mono-infected male) or totally (bi-infected male) the molecules in question.

3. *Slow-motion hypothesis.* If the actions of different slowing down factors are additive, then an incoming paternal DNA bearing only factor A could be provided with factor B by the *Wolbachia* present in the egg, and be synchronized with maternal DNA affected by A and B.

F. mod and resc are functionally independent: the [mod− resc+] phenotype does exist

The *mod resc* notation[4] allows CI *Wolbachia* to be classified in four theoretical phenotypic categories: (i) [*mod*+ *resc*+], the ''invasive'' phenotype, where *Wolbachia* induces CI and rescues from it, (ii) [*mod*− *resc*−], the ''helpless'' phenotype, where *Wolbachia* is unable to induce CI nor to rescue from it, (iii) [*mod*+ *resc*−], the ''suicide'' phenotype, where *Wolbachia* is able to induce CI but unable to rescue its own effect and

(iv) [*mod*– *resc*+], the ''defensive'' phenotype, where *Wolbachia* rescues CI from at least one type of *mod*+ variant, but is unable itself to induce CI. The [*mod*+ *resc*–] ''suicide'' phenotype has never been observed, but theory does not preclude its existence.[22,23] On the contrary, the three other types have been found in the wild: [*mod*+ *resc*+],[24] [*mod*– *resc*–][25] and [*mod*– *resc*+].[7,26,27] The existence of this latter type demonstrates that *mod* and *resc* are functionally independent: *resc* can remain fully efficient while *mod* has been lost. How do the different models account for this finding ?

1. *Lock-and-key hypothesis.* *mod* and *resc* are controlled by different genes. Thus, the emergence of *mod*– *resc*+ mutants can indeed be expected.

2. *Titration–restitution hypothesis.* One important aspect of this model is that the *resc* function is subordinated to a functional *mod*: obviously, *Wolbachia* can only give back host molecules that they are able to capture and store beforehand. Thus, *mod* must be functional prior to fertilization for *resc* to take place after fertilization. One possibility to circumvent this difficulty would be that in [*mod*– *resc*+] strains, *mod* (titration) is expressed in female hosts (allowing subsequent restitution) but not in males. Thus, the additional hypothesis of a sex-specific expression of *mod* is necessary.

3. *Slow-motion hypothesis.* This model postulates that *mod* and *resc* are determined by the same gene(s). The existence of the [*mod*– *resc*+] phenotype thus requires a similar additional hypothesis as above: the slowing down factor would be expressed in females, but not in males.

G. Different mod resc pairs have most probably evolved from a common ancestor

Although this point is not a demonstrated fact, both the number of different and bi-directionally incompatible variants and the existence of partially compatible variants make it the most parsimonious hypothesis. Let us consider an ancestral variant $mod_A resc_A$ and how it could evolve into a new, bi-directionally incompatible variant $mod_B resc_B$.

1. *Lock-and-key hypothesis.* Since the lock-and-key hypothesis implies that *mod* and *resc* are controlled by different genetic determinants, the new $mod_B resc_B$ could appear through an intermediate $mod_B resc_A$ stage, following a process described in a recent theoretical paper.[23]

2. *Titration–restitution hypothesis.* An intermediate $mod_B resc_A$ type cannot occur here, because one can restore only what has been titrated. Therefore, any mutation from mod_A to mod_B also means that $resc_A$ becomes $resc_B$. Such a new $mod_B resc_B$ mutant would then face the very difficult task of invading an incompatible $mod_A resc_A$ population, where it would be strongly outnumbered and thus selected against.[28,29] However, if we make the additional hypothesis that A and B types differ very slightly (weak bi-directional incompatibility), and if host populations are small, the $mod_B resc_B$ type might get fixed by drift, just as any slightly deleterious mutation. The new variant would of course invade the population more easily if it was associated with positive effects on host physiology or if its rate of maternal transmission was higher than that of the previous variant.[30]

3. *Slow-motion hypothesis.* Under this model, *mod* and *resc* are controlled by the same gene, so that the $mod_B resc_A$ type cannot occur. The conditions for the emergence of new compatibility types from ancestral ones are thus as stringent as described above in the case of the titration–restitution model.

Conclusions

It follows from this comparative analysis of CI models that the titration–restitution and slow-motion models account for most observations, but require however the additional hypothesis that the [*mod*– *resc*+] phenotype is due to a sex-specific expression of bacterial genes. In addition, they would also seem to impose serious constraints on the evolution of new CI types. In contrast, the lock-and-key model accounts for all the facts known to date, and thus remains, in theory, the most parsimonious CI model currently available. Yet, it only requires a tiny but solid fact to bring down the nicest theoretical edifice, and given the accelerating pace of *Wolbachia* research, we might not have very long to wait for the solution of this half-century-old riddle.

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