

Experimental replacement of an obligate insect symbiont

Nancy A. Moran^{a,1} and Yueli Yun^{a,b}

^aDepartment of Integrative Biology, University of Texas at Austin, Austin, TX 78712; and ^bCentre for Behavioural Ecology and Evolution, College of Life Sciences, Hubei University, Wuhan 430062, Hubei, China

Edited by Joan E. Strassmann, Washington University in St. Louis, St. Louis, MO, and approved November 24, 2014 (received for review October 19, 2014)

Symbiosis, the close association of unrelated organisms, has been pivotal in biological diversification. In the obligate symbioses found in many insect hosts, organisms that were once independent are permanently and intimately associated, resulting in expanded ecological capabilities. The primary model for this kind of symbiosis is the association between the bacterium *Buchnera* and the pea aphid (*Acyrtosiphon pisum*). A longstanding obstacle to efforts to illuminate genetic changes underlying obligate symbioses has been the inability to experimentally disrupt and reconstitute symbiont–host partnerships. Our experiments show that *Buchnera* can be experimentally transferred between aphid matrilines and, furthermore, that *Buchnera* replacement has a massive effect on host fitness. Using a recipient pea aphid matriline containing *Buchnera* that are heat sensitive because of an allele eliminating the heat shock response of a small chaperone, we reduced native *Buchnera* through heat exposure and introduced a genetically distinct *Buchnera* from another matriline, achieving complete replacement and stable inheritance. This transfer disrupted 100 million years (~1 billion generations) of continuous maternal transmission of *Buchnera* in its host aphids. Furthermore, aphids with the *Buchnera* replacement enjoyed a dramatic increase in heat tolerance, directly demonstrating a strong effect of symbiont genotype on host ecology.

symbiosis | aphid | *Buchnera* | thermal tolerance | maternal transmission

Symbiosis has been key in the ecological and evolutionary diversification of eukaryotes (1, 2). In many invertebrates, bacterial symbionts have been maternally transmitted for millions of years and are required for the growth and reproduction of hosts (3). These symbionts approach organelles in their degree of genetic and physiological integration with hosts and in their extreme genomic reduction. A model for obligate symbiosis is that of the pea aphid (*Acyrtosiphon pisum*) and its nutrient-provisioning bacterial symbiont, *Buchnera aphidicola*. *Buchnera* features a tiny genome (4), restriction to a small number of specialized host cells (bacteriocytes), host-controlled transmission (5), and regulated exchange of molecules with hosts (6, 7). This tight integration creates challenges for studies that aim to elucidate how symbiont variation affects host fitness and ecology.

The ability to transfer obligate symbionts between host matrilines could provide a tool for teasing apart the separate contributions of symbiotic partners. Facultative symbionts, such as those conferring defense against pathogens, have been transferred experimentally into novel host matrilines, where they are typically stably inherited, enabling direct measures of symbionts on hosts (8). The success of these transfers is presumably related to the fact that facultative symbionts possess their own machinery for invading host cells (9, 10) and typically persist in several locations in the insect body. In contrast, obligate symbionts such as *Buchnera* are packaged into specialized host cells during early development and do not survive in the hemocoel or in other cell types.

Buchnera colonizes developing aphids before birth, through a specialized transmission process that has been studied in detail in *A. pisum* (5, 11). Aphids are parthenogenetic for much of their life cycle, during which embryos develop within maternal ovarioles. Bacteriocytes and ovarioles containing developing embryos

are located near one another within the mother's abdomen, and transmission occurs when *Buchnera* cells are exocytosed from a maternal bacteriocyte in the vicinity of a blastula-stage embryo (5). The *Buchnera* cells become extracellular within the hemocoel, and some are endocytosed by the posterior syncytial cytoplasm of the embryo in which they are later packaged into the embryonic bacteriocytes. *Buchnera* cells released into the hemocoel quickly deteriorate if they do not enter an embryo. The molecular underpinnings of the transfer process are unknown. This specialized transmission process presents a challenge for the experimental transfer of symbionts between hosts.

We developed a strategy aimed at replacing resident *Buchnera* with genetically distinct *Buchnera* from a different host matriline (Fig. 1), using parthenogenetic (all-female) lines of aphids. We used heat tolerance as a selectable phenotype. A previous study showed that a single nucleotide deletion in the promoter of a small heat shock protein [inclusion body-associated protein A (IbpA)] of *Buchnera* results in a reduction in *Buchnera* numbers of >100-fold after exposure to 4 h of 35 °C heat (12). *Buchnera* lacking this mutation undergo only modest declines after heat exposure. Our strategy was to reduce the native *Buchnera* in recipient aphids using heat, and then to inject homogenate containing heat-tolerant *Buchnera* from another *A. pisum* matriline. Because transmission to embryos includes an extracellular stage in which *Buchnera* is free in the hemocoel, the injected *Buchnera* might colonize embryos in lieu of maternal *Buchnera*, which have been eliminated or depleted. After successful replacement of native *Buchnera*, we demonstrated a large effect on aphid ability to withstand heat exposure.

Significance

In many invertebrates, development depends on obligate bacterial symbionts that are confined to specialized host cells and are transmitted directly from mother to progeny. A primary model for this kind of symbiosis is the *Buchnera*/pea aphid association, which has been maintained for more than 100 million years through strict maternal transmission. The intimacy of this symbiosis has thwarted experiments aimed at dissecting how symbiont and host genotypes contribute to overall phenotype and ecological tolerances. Using a selectable *Buchnera* phenotype combined with microinjection, we successfully replaced *Buchnera* within a matriline, thus generating matrilines with identical aphid genotypes but distinct *Buchnera* genotypes. *Buchnera* replacement dramatically increased heat tolerance of the aphid matriline, demonstrating directly that symbiont genotype can affect host ecology.

Author contributions: N.A.M. designed research; N.A.M. and Y.Y. performed research; N.A.M. contributed new reagents/analytic tools; N.A.M. and Y.Y. analyzed data; and N.A.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

See Commentary on page 1923.

¹To whom correspondence should be addressed. Email: nancy.moran@austin.utexas.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1420037112/-DCSupplemental.

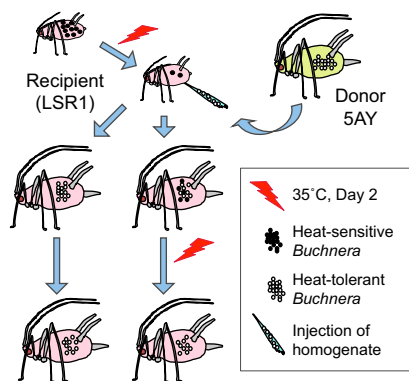


Fig. 1. Experimental approach for replacement of the native *Buchnera* symbionts within an *A. pisum* matriline. The recipient line (LSR1) contains a heat-sensitive *Buchnera* genotype, and the donor (5AY) contains a heat-tolerant *Buchnera* genotype. Native *Buchnera* are depleted by heat in the recipient line, and microinjection is used to flood the hemocoel with donor *Buchnera*. Most embryos are successfully colonized by the donor symbionts. In some cases, complete replacement occurs in the progeny of injected females. In other cases, progeny have a mixed *Buchnera* population, which can be shifted completely to the donor type through further heat exposure.

Results

Replacement of Native Symbionts with Donor Symbionts. We screened several parthenogenetic *A. pisum* lines for the *Buchnera* allele affecting heat tolerance. *A. pisum* LSR1 was chosen as the recipient matriline; this aphid clone was used for full-genome sequencing by the International Aphid Genome Consortium and had acquired the *Buchnera* mutation conferring heat sensitivity in our laboratory during the last 5 y. *A. pisum* 5AY, containing the heat-tolerant *Buchnera* allele, was selected as the donor. After heat exposure (4 h at 35 °C) on day 2 after birth, LSR1 juveniles were injected on day 3, using homogenate from 5AY. Two sets of injections were carried out, with 25–30 aphids per set. Most injected individuals died within 24 h from the combined trauma of the heat exposure and the injection. Those that survived the first 24 h usually became adult and reproduced, although ages at which they reached adulthood and first reproduced were considerably delayed. Under our conditions, normal *A. pisum* require 7 d from birth to adulthood and 8 d from birth to first reproduction, but heat-treated and injected females were as old as 15 d on reaching adulthood and as old as 22 d at first reproduction.

In set 1, four females survived to adulthood, and all four reproduced. In set 2, six females survived to adulthood and five reproduced. For each injected female that reproduced, progeny were sampled to check for the presence of the two *Buchnera* types. A genetic marker distinct from the locus affecting heat tolerance was used for screening.

Of the nine injected females that matured and reproduced, the donor (5AY) *Buchnera* was present in the F1 progeny of eight, either mixed with the LSR1 *Buchnera* or as the sole *Buchnera* type (Table 1). In set 1, all four females produced progeny containing a mixture of donor and recipient *Buchnera* types (Fig. 2). One of the four matrilines was propagated, and F2, F3, and F4

progeny also contained mixed *Buchnera* populations (Fig. 2). To achieve complete replacement with the 5AY *Buchnera* in this matriline, 2-d-old F5 nymphs were subjected to a further heat treatment, resulting in the exclusive presence of the 5AY *Buchnera* type in the F6 generation. Subsequent generations retained exclusively 5AY *Buchnera* (Fig. 2). In set 2, two mothers produced F1 progeny in which the donor had completely replaced the native *Buchnera* (Table 1, Fig. 2). Of the other three adults of set 2, two produced progeny with mixed *Buchnera* populations, as in set 1, and one produced progeny with only the native LSR1 *Buchnera*. Thus, although individual injections differed in outcome, this protocol gave a high frequency of transfected lines.

Effects of Symbiont Replacement on Host Thermal Tolerance. After the successful replacement of the heat-sensitive LSR1 *Buchnera* with heat-tolerant 5AY *Buchnera*, we examined the consequences for aphid growth and reproduction at different temperatures. We compared developmental time, adult mass, and fecundity of *A. pisum* LSR1 aphids retaining native *Buchnera* with those of *A. pisum* LSR1 aphids with the 5AY *Buchnera* under constant 20 °C and under 20 °C interrupted by exposure to 35 °C for 4 h on day 2 (*SI Methods*). Comparing across treatment categories, there were highly significant differences in all fitness measures: developmental time (Kruskal-Wallis, $H = 59.7$; $df = 3$; $P < 0.0001$), adult weight (Kruskal-Wallis, $H = 111.3$; $df = 3$; $P < 0.0001$), and fecundity (Kruskal-Wallis, $H = 35.8$; $df = 3$; $P < 0.0001$; Fig. 3). These differences were entirely attributable to lower fitness of *A. pisum* LSR1 with native *Buchnera* after heat: These aphids took longer to mature, were smaller, and had lower fecundity compared with other treatment categories. In contrast, *A. pisum* LSR1 with the replacement 5AY *Buchnera* were not significantly affected by heat exposure, for any of the measures, and the two lines had similar performance, with no significant differences between them, under constant 20 °C (Fig. 3). Thus, the replacement of its *Buchnera* greatly increased heat tolerance of the *A. pisum* LSR1 aphid matriline, demonstrating a major effect of symbiont genotype on host fitness.

Discussion

In parthenogenetic aphid generations, embryos begin development before their mothers are mature, so embryos ready for colonization are present in the injected juvenile aphids. The success of the transfers indicates an ability of the donor *Buchnera* to invade the posterior syncytium of these developing embryos and to be packaged normally during subsequent development and inherited stably in subsequent generations. The heat-induced depletion of maternal *Buchnera* confers a numerical advantage to donor *Buchnera*. In many cases, the initial injection resulted in a mixed population of *Buchnera*, and further selection with heat resulted in complete replacement.

A limitation of this method is that the recipient matriline must contain *Buchnera* with the *ibpA* allele that confers heat intolerance. However, we have found that *Buchnera* in most *A. pisum* matrilines evolve to the heat-sensitive genotype after extended laboratory culture at 15–20 °C, reflecting a high mutation rate at this allele and selection favoring this genotype under cool conditions (12).

The persistence of both *Buchnera* types for four generations, before the replacement was completed through a second round of selection with heat, is somewhat unexpected; it suggests that

Table 1. Experimental replacement of native *Buchnera* (LSR1 type) with donor *Buchnera* (5AY type)

Set	Heat treatment	Number injected that survive to adulthood	Number injected that reproduce	Number surviving mothers with F1 having:		
				Mixed <i>Buchnera</i>	Only LSR1 <i>Buchnera</i>	Only 5AY <i>Buchnera</i>
1	4 h, 35 °C	4	4	4	0	0
2	5 h, 35 °C	6	5	2	1	2

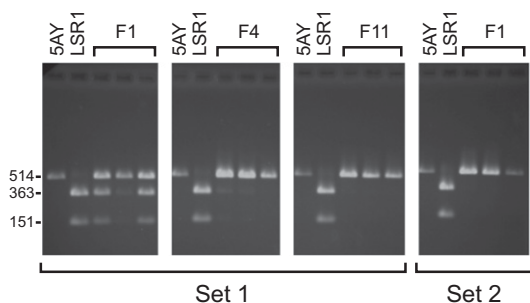


Fig. 2. Screens for the presence of the LSR1 and 5AY *Buchnera* genotypes based on a PCR and diagnostic restriction digest. Each lane is from a screen of a single female aphid; lanes labeled 5AY and LSR1 are controls from stock aphids. LSR1 *Buchnera* contain a distinctive restriction site, yielding bands of 363 bp and 151 bp for LSR1 and a single band of 514 bp for 5AY. Results for set 1 are shown for the F1, F4, and F11 generations. Some LSR1 *Buchnera* remained in F4 females but were eliminated by a heat exposure in F5, and this was maintained in future generations, as shown by the assay in F11. In set 2, F1 individuals exhibited complete replacement of the original LSR1 *Buchnera* by the 5AY *Buchnera*.

the effective bottleneck size is large enough to enable persistence of polymorphism across generations. A previous estimate of the number of *Buchnera* infecting sexual progeny was 1,800 (13); if the number is similar for parthenogenetic generations, it would be consistent with the maintenance of polymorphism of equally fit *Buchnera* over numerous generations. This question of bottleneck size is complicated by several aspects of the symbiont life cycle: they divide rapidly around the time of inoculation, are packaged into bacteriocytes during development, and can colonize an embryo from more than one maternal bacteriocyte. The effective bottleneck size could be estimated by assaying the rate of loss of *Buchnera* polymorphism over aphid generations, which is a potential application of our method of *Buchnera* transfer.

In addition to obligate symbionts, exemplified by *Buchnera*, insects including aphids often contain facultative bacterial symbionts that are mostly maternally transmitted but undergo occasional horizontal transfer between host matriline (14, 15). Many facultative symbionts are readily transferred between hosts in the laboratory, and such transfers have been a critical tool in the experimental identification of symbiont-based traits while controlling for host genetic background (8, 15–19). This ease of transferring facultative symbionts might be expected, as they undergo horizontal transfer in natural populations via several mechanisms (20–22), and their genomes encode machinery such as type III secretion systems, known to enable invasion of eukaryotic cells (9, 10). In contrast, exclusively maternally transmitted, obligate symbionts, such as *Buchnera*, are never faced with the challenge of invading new matriline. These obligate symbionts have reduced genomes that do not encode recognizable mechanisms for host cell invasion (23). Microscopy studies suggest that *Buchnera* is much more dependent than facultative symbionts on host-based mechanisms for transmission from mother to progeny (5).

An alternative approach to assigning phenotypic traits to host versus symbiont is to create specific combinations of symbiont and host genotypes through genetic crosses that exploit the exclusive maternal transmission of symbionts (24, 25). However, genetic crosses are difficult or impossible in many host species, including aphids. Our method generates transfected insects with identical host genotypes within several weeks and enables examination of the effects of *Buchnera* genotype while controlling for aphid genetic background. Potentially, the approach could be combined with mutagenesis (26) to explore effects of *Buchnera* mutations on the symbiont–host interface.

The intimate interdependence of *Buchnera* and aphids approaches that of organelles and their host cells; for example, it

involves exchange of molecules, including host-encoded proteins between the host and bacterial cytosols (7). An unanswered question is the extent of reciprocal coadaptation of host and symbiont lineages. Potentially, the rapid evolution of symbiont genes and genomes (23) is tracked by adaptive compensatory changes in host genes, accelerating the establishment of incompatibilities between symbiont and host genotypes from different host matriline. If so, intimate symbioses might facilitate reproductive isolation and speciation. If beneficial symbionts such as *Buchnera* are generally interchangeable among related host species, this would suggest the lack of an evolutionary “arms race” in these mutualistic relationships. Transfers of *Buchnera* between aphids with different levels of divergence could be useful for exploring the extent of symbiont–host genetic matching and for illuminating the nature of the symbiotic interface.

Methods

Replacement of Native *Buchnera*. After screening laboratory aphid lines for the *Buchnera* allele that governs heat sensitivity (*SI Methods*), we selected as recipient *A. pisum* LSR1, previously used for genome sequencing of *A. pisum* (27). This line had gained the heat-sensitive *Buchnera* mutation during long-term laboratory culture. As donor, we selected *A. pisum* 5AY, which was previously isolated as a subline of *A. pisum* 5A (28), for which the *Buchnera* genome has been sequenced (29). *A. pisum* 5AY contained *Buchnera* with the heat-tolerant allele. We developed an assay to distinguish the two *Buchnera* types, based on a polymorphism distinct from that affecting heat tolerance that introduced a unique restriction site into the LSR1 fragment. Thus, the assay consisted of PCR amplification and restriction digestion of a 514-base pair genomic fragment containing the polymorphism, followed by agarose gel electrophoresis (details in *SI Methods*).

To reduce *Buchnera* numbers in recipients, 2-d-old nymphs of *A. pisum* LSR1 were heat-exposed at 34–35 °C for 4 (set 1) or 5 (set 2) h. In each set, 25–30 individuals were injected the following day with homogenate consisting of freshly crushed abdomens of fourth instar or young (1–3 d post-molt) adults of the *A. pisum* 5AY donor line. Injected females were placed on plants to feed. Two different investigators performed the two sets of injections. Progeny of the injected females were allowed to mature and

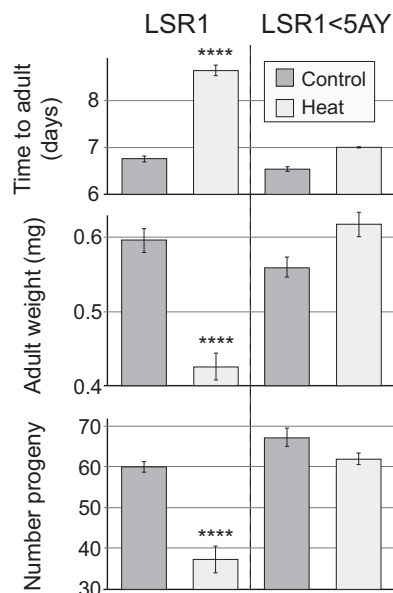


Fig. 3. Effect of symbiont replacement on aphid fitness under constant 20 °C and after heat exposure, measured as developmental time, adult weight, and fecundity (mean \pm SE). The LSR1 matriline contains heat-sensitive *Buchnera*, whereas the LSR1 < 5AY matriline has a replacement *Buchnera* with normal heat tolerance. Replacement of *Buchnera* results in faster development, larger adult size, and increased fecundity after heat stress (**** P < 0.0001, Kruskal-Wallis).

reproduce before being collected for screening, and screening was continued for up to 11 generations.

For cases of coexistence of both *Buchnera* genotypes within a matriline, 2-d-old females were again exposed to 4 h of 34–35 °C, and their descendants were allowed to mature and reproduce before being screened.

Measurement of *Buchnera* Effect on Aphid Thermal Tolerance. Heat tolerance of a line of LSR1 < 5AY (*A. pisum* LSR1 in which the original *Buchnera* had been replaced with *Buchnera* of *A. pisum* 5AY) was compared with the heat tolerance of the original line of *A. pisum* LSR1. The LSR1 < 5AY line was from the set 1 transfer experiment and was compared using aphids from generation 12 after the transfer. Adults of LSR1 and of LSR1 < 5AY were placed on plants overnight at 20 °C and removed, resulting in even-aged nymphs on each plant. Eight colonies were established for each of the two matrilines. On the following day, four colonies of each line were placed at 35 °C for 4 h

and then returned to 20 °C. Four control colonies of each line were kept at continuous 20 °C. Aphids were monitored daily, and dates of molting to adulthood and weights of new adults were recorded, with total sample sizes of 60, 48, 59, and 84 aphids for LSR1 heat-treated, LSR1 control, LSR1 < 5AY heat-treated, and LSR1 < 5AY control, respectively. For each of the 16 colonies, four newly eclosed adults were placed individually on fresh plants, and these were kept at 20 °C. Date of first reproduction was recorded, and total number of nymphs produced during the following 6 d was recorded as a measure of fecundity.

ACKNOWLEDGMENTS. We thank Howard Ochman for running electrophoretic gels and Kim Hammond for help with growing plants and aphids. Y.Y. was supported by a fellowship from the China Scholarship Council, and other research funding was received from the University of Texas at Austin.

1. Archibald J (2014) *One Plus One Equals One: Symbiosis and the Evolution of Complex Life* (Oxford University Press, Oxford).
2. McFall-Ngai M, et al. (2013) Animals in a bacterial world, a new imperative for the life sciences. *Proc Natl Acad Sci USA* 110(9):3229–3236.
3. Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. *Annu Rev Genet* 42:165–190.
4. Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H (2000) Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. *APS. Nature* 407(6800): 81–86.
5. Koga R, Meng X-Y, Tsuchida T, Fukatsu T (2012) Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. *Proc Natl Acad Sci USA* 109(20):E1230–E1237.
6. Price DRG, et al. (2014) Aphid amino acid transporter regulates glutamine supply to intracellular bacterial symbionts. *Proc Natl Acad Sci USA* 111(1):320–325.
7. Nakabachi A, Ishida K, Hongoh Y, Ohkuma M, Miyagishima SY (2014) Aphid gene of bacterial origin encodes a protein transported to an obligate endosymbiont. *Curr Biol* 24(14):R640–R641.
8. Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu Rev Entomol* 55: 247–266.
9. Degnan PH, Yu Y, Sisneros N, Wing RA, Moran NA (2009) *Hamiltonella defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. *Proc Natl Acad Sci USA* 106(22):9063–9068.
10. Dale C, Plague GR, Wang B, Ochman H, Moran NA (2002) Type III secretion systems and the evolution of mutualistic endosymbiosis. *Proc Natl Acad Sci USA* 99(19):12397–12402.
11. Braendle C, et al. (2003) Developmental origin and evolution of bacteriocytes in the aphid-*Buchnera* symbiosis. *PLoS Biol* 1(1):E21.
12. Dunbar HE, Wilson ACC, Ferguson NR, Moran NA (2007) Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. *PLoS Biol* 5(5):e96.
13. Mira A, Moran NA (2002) Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. *Microb Ecol* 44(2):137–144.
14. Sandström JP, Russell JA, White JP, Moran NA (2001) Independent origins and horizontal transfer of bacterial symbionts of aphids. *Mol Ecol* 10(1):217–228.
15. Boyle L, O'Neill SL, Robertson HM, Karr TL (1993) Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. *Science* 260(5115):1796–1799.
16. Montllor CB, Maxmen A, Purcell AH (2002) Facultative bacterial endosymbionts benefit pea aphids *Acyrtosiphon pisum* under heat stress. *Ecol Entomol* 27: 189–195.
17. Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc Natl Acad Sci USA* 100(4):1803–1807.
18. Rouchet R, Vorburger C (2012) Strong specificity in the interaction between parasitoids and symbiont-protected hosts. *J Evol Biol* 25(11):2369–2375.
19. Walker T, et al. (2011) The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* 476(7361):450–453.
20. Moran NA, Dunbar HE (2006) Sexual acquisition of beneficial symbionts in aphids. *Proc Natl Acad Sci USA* 103(34):12803–12806.
21. Gehrler L, Vorburger C (2012) Parasitoids as vectors of facultative bacterial endosymbionts in aphids. *Biol Lett*, 10.1098/rsbl.2012.0144.
22. Huigens ME, et al. (2000) Infectious parthenogenesis. *Nature* 405(6783):178–179.
23. McCutcheon JP, Moran NA (2012) Extreme genome reduction in symbiotic bacteria. *Nat Rev Microbiol* 10(1):13–26.
24. Vogel KJ, Moran NA (2011) Sources of variation in dietary requirements in an obligate nutritional symbiosis. *Proc Biol Sci* 278(1702):115–121.
25. Jaenike J (2007) Spontaneous emergence of a new *Wolbachia* phenotype. *Evolution* 61(9):2244–2252.
26. Tagu D, Le Trionnaire G, Tanguy S, Gauthier J-P, Huynh J-R (2014) EMS mutagenesis in the pea aphid *Acyrtosiphon pisum*. *G3 (Bethesda)* 4:657–667.
27. International Aphid Genomics Consortium (2010) Genome sequence of the pea aphid *Acyrtosiphon pisum*. *PLoS Biol* 8(2):e1000313.
28. Moran NA, Jarvik T (2010) Lateral transfer of genes from fungi underlies carotenoid production in aphids. *Science* 328(5978):624–627.
29. Moran NA, McLaughlin HJ, Sorek R (2009) The dynamics and time scale of ongoing genomic erosion in symbiotic bacteria. *Science* 323(5912):379–382.