Supporting Online Material for

Symbiotic Bacterium Modifies Aphid Body Color

Tsutomu Tsuchida,* Ryuichi Koga, Mitsuyo Horikawa, Tetsuto Tsunoda, Takashi Maoka, Shogo Matsumoto, Jean-Christophe Simon, Takema Fukatsu*

*To whom correspondence should be addressed. E-mail: t-tsuchida@riken.jp (T.T.); t-fukatsu@aist.go.jp (T.F.)

Published 19 November 2010, *Science* **330**, 1102 (2010)
DOI: 10.1126/science.1195463

This PDF file includes:

Symbiotic Bacterium Modifies Aphid Body Color

Tsutomu Tsuchida, Ryuichi Koga, Mitsuyo Horikawa, Tetsuto Tsunoda, Takashi Maoka, Shogo Matsumoto, Jean-Christophe Simon, Takema Fukatsu

Supporting Online Material

Materials and methods

Insects. Pea aphids, *Acyrthosiphon pisum,* were collected in Western Europe from 2006 to 2008. Large, putative adult insects were sampled from various legume plants, and their body color was recorded upon collection. Details on geographic and plant origins are available upon request to JCS. Pea aphid strains were maintained on seedlings of the broad bean, *Vicia faba*, at 20^{o}C in a long day regime (16 h light: 8 h dark). Genotypic identity of each strain was assessed by genetic markers and regularly checked (*S1*).

Phylogenetic analysis and diagnostic PCR. Screening of symbiotic bacteria was conducted as described (*S2*). 16S rRNA gene sequences of the *Rickettsiella* endosymbionts were deposited in the DNA Data Bank of Japan (accession nos. AB522697-AB522705). PCR detection of *Rickettsiella* was conducted using specific primers targeting 16S rRNA gene, RCL16S-211F (5'-GGG CCT TGC GCT CTA GGT-3') and RCL16S-470R (5'-TGG GTA CCG TCA CAG TAA TCG A-3') under a temperature profile of 35 cycles consisting of 95^{o}C for 30 sec, 55^{o}C for 30 sec and 72^{o}C for 1 min. Diagnostic PCR of other symbiotic bacteria were conducted as described (*S3*). A maximum likelihood phylogeny with bootstrap values was inferred as described (*S4*).

Endosymbiont elimination and transfection. Selective elimination of *Hamiltonella* was conducted by injecting a mixture of antibiotics ampicillin, cefotaxime and gentamycin, each at a dose of 250 μg/ml as described (*S5*). Selective elimination of *Serratia* was performed by ampicillin injection as described (*S6*). We repeatedly attempted different cocktails and doses of the antibiotics, but failed to eliminate *Rickettsiella* without affecting *Buchnera* infection. Transfection of *Rickettsiella* was conducted by hemolymph injection (*S6*). All the recipient aphid strains were treated in advance with the antibiotics to eliminate pre-existing facultative endosymbionts, and successful elimination was confirmed by diagnostic PCR. Newborn nymphs deposited by the injected mothers were collected 11 days after injection and reared individually, from which isofemale lines were established. *Rickettsiella* infections in the isofemale

lines were checked by diagnostic PCR for three successive generations.

Evaluation of aphid body color. Twelve 11-day-old insects (four insects x three successive generations) per strain were analyzed. Aphids were anesthetized with carbon dioxide and placed on a filter paper, and their color images were taken by a digital camera (EC3 Leica) connected to a microscope (S8APO, Leica). The images were analyzed using Adobe Photoshop CS software (version 8.01). Ten pixels were randomly chosen from the abdomen of each insect, hue angles were inferred for each of them, and the average of the hue angles was defined as the index of body color.

Quantitative PCR. *Rickettsiella gyrB* gene copies and aphid *elongation factor 1α* (*ef1α*) gene copies were quantified using SYBR green and Mx3000P QPCR system (Agilent) as described (*S7*) with primers RclGyrB-AF1 (5′-GAG GCA CTG AAA TCC GCT TTT ATC C-3′) and RclGyrB-AR1 (5′-GGC AAC GCC AGA ATT TAG GAA TGA G-3′) for *gyrB* and with primers ApisEF-AF1 (5'-CTG GAG AAT TCG AAG CTG GTA TTT-3') and ApisEF-AR1 (5′-CAC CCA AGG TGA AAG CCA ATA G-3′) for *ef1α*.

Histology. *Rickettsiella* and *Buchnera* in aphid embryos were visualized by whole-mount fluorescent *in situ* hybridization as described (*S8*) with an AlexaFluor555-labeled probe Al555-RCL1252R (5'-TCG CGG GTT GGC TTC CT-3') and a Cy5-labeled probe Cy5-ApisP2a (5'-CCT CTT TTG GGT AGA TCC-3') targeting their 16S rRNA, respectively. Transmission electron microscopy was conducted as described (*S4*).

Fitness measurement. Each newborn nymph was placed on a potted seedling of the broad bean, and reared at 20^{o}C in a long day regime (16 h light: 8 h dark). The plant was renewed every week. For each insect, fresh body weight on the 8th day, time to first reproduction, total number of offspring, and longevity were recorded.

Carotenoid analysis. About 300-400 mg of each aphid strain was extracted with 5 ml of acetone three times at room temperature. The extracted solutions were combined and filtrated, and the total amount of carotenoids was evaluated as described (*S9*). Then the solution was evaporated to dryness and dissolved in *t*-butyl methyl ether: methanol: water (48: 48: 4) and subjected to high performance liquid chromatography (HPLC) analysis under the following conditions: column, YMC Carotenoid column (250 x 4.5 mm i.d., 5 μm, Waters Corporation MA USA); solvent A, *t*-butyl methyl ether: methanol: water (48: 48: 4); solvent B, *t*-butyl methyl ether; gradient system, 0~10 min, solvent A 100%; 10~20 min, solvent B

0%~100 % linear gradient; 20min~45min, solvent B 100%; flow rate, 1.0 min/ml; detection, 470 nm. In the HPLC analysis, the relative amounts of the carotenoids were calculated from the peak area detected at 470 nm. Identification of each of the carotenoids was based on retention time in HPLC and VIS absorption spectra.

Quantitative reverse transcription-PCR. RNA was isolated from 11-day-old adult aphids using TRIzol reagent, followed by RNase-free DNase I treatment. First strand cDNAs were synthesized using pd(N)6 primer and PrimeScript reverse transcriptase (Takara). Quantification was performed as described (*S10*) using the ABI PRISM 7000 instrument and THUNDERBIRD SYBR qPCR Mix (Toyobo) with primers LOC100169245_1123F (5'-ATC CGA CAG CAG CCC CCG AA-3') and LOC100169245_1465R (5'-GTA CCC GGG TGC GCA GAA GC-3') for *tor* gene, the fungus-derived carotenoid desaturase gene responsible for production of red carotenoids in the pea aphid (*S11*). The relative expression levels were normalized to the transcript levels of the ribosomal protein *RpL7* and statistical analyses were performed as described (*S10*).

Thin layer chromatography of aphid pigments. Around 15 mg of frozen aphids were squashed by a spatula and extracted with 100 μl of *n*-hexane twice. After the solution was absorbed with a small piece of soft paper to remove lipids, each insect residue was extracted with a mixture of 150 μl water and 150 μl *n*-butanol, and washed with 200 μl of *n*-butanol. Almost all green pigments were yielded in the pooled *n*-butanol fraction (350 μl in total), of which 200 μl (equivalent to 8.6 mg of insects) was taken and evaporated. The resultant residue was dissolved in 15 μl of methanol, and subjected to reverse phase thin layer chromatography (TLC) on pre-coated silica gel plates (60 RP-18 WF254S, Merck) using water-methanol (1: 2) as solvent. Under the condition, polycyclic quinone glycosides like aphinins are efficiently separated on the plates, while carotenoids and other hydrophobic pigments do not migrate. The TLC images were scanned with EPSON GT-X800 and analyzed by Adobe Photoshop CS software. Densitometric quantification of the major band was performed by Image J software (version 1.43).

References

S1. J. Peccoud, A. Ollivier, M. Plantegenest, J. C. Simon, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 7495 (2009).

S2. T. Fukatsu, N. Nikoh, *Appl. Environ. Microbiol*. **64**, 3599 (1998).

S3. T. Tsuchida, R. Koga, H. Shibao, T. Matsumoto, T. Fukatsu, *Mol. Ecol.* **11**, 2123 (2002).

S4. H. Toju *et al*., *Appl. Environ. Microbiol*. **76**, 275 (2010).

S5. A. E. Douglas *et al*., *Physiol. Entomol.* **31**, 262 (2006).

S6. R. Koga, T. Tsuchida, M. Sakurai, T. Fukatsu, *FEMS Microbiol. Ecol*. **60**, 229 (2007).

S7. S. Narita, D. Kageyama, M. Nomura, T. Fukatsu*, Appl. Environ. Microbiol*. **73**, 4332 (2007).

S8. R. Koga, T. Tsuchida, T. Fukatsu, *Appl. Entomol. Zool.* **44**, 281 (2009).

S9. K. Schiedt, S. Liaaen-Jensen, in Carotenoids, G. Britton, S. Liaaen-Jensen, H. Pfander Eds. (Birkhäuser, Basel, 1995), Vol. 1A, pp 81-108.

S10. A. Nakabachi *et al*., *Proc. Natl. Acad. Sci. U.S.A.* **102**, 5477-5482 (2005).

S11. N. A. Moran, T. Jarvik, *Science* **328**, 624 (2010).

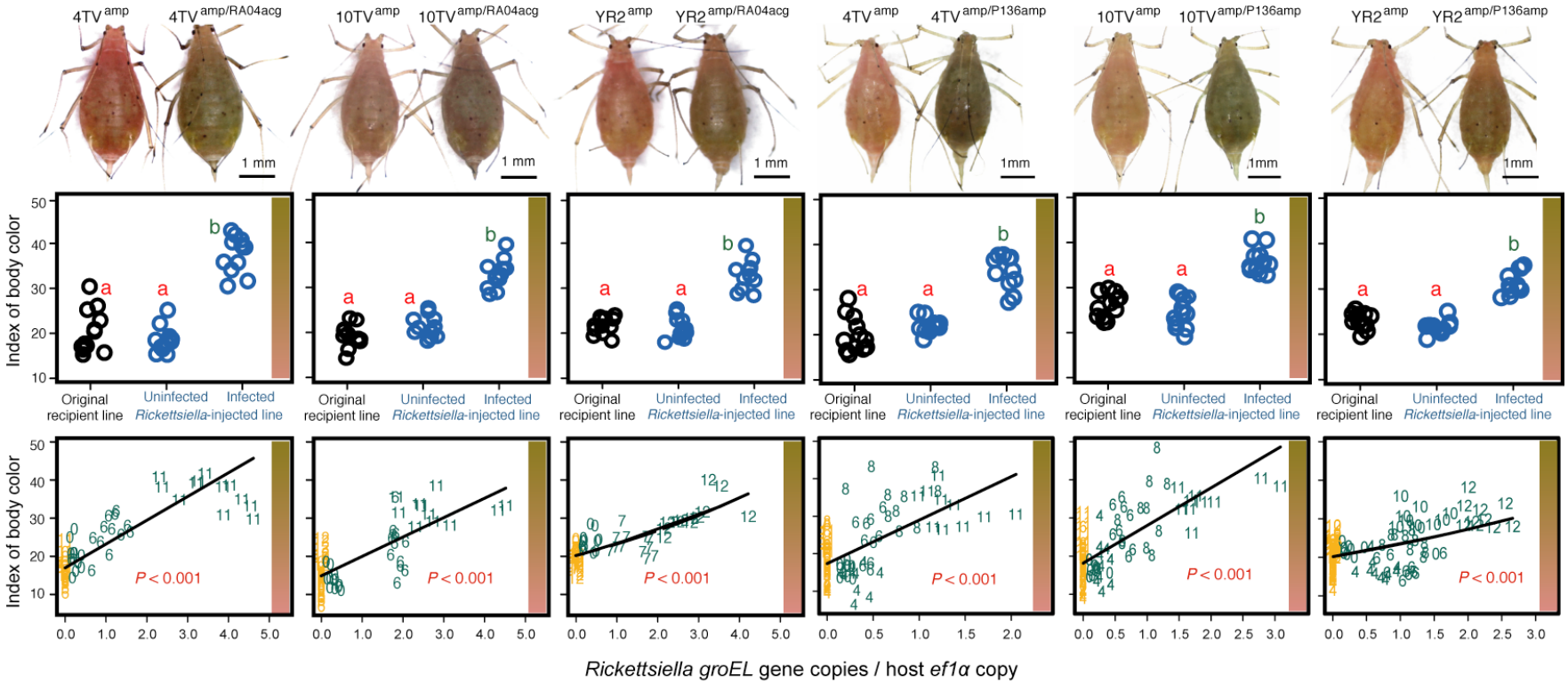

Fig. S1. (Top panels) A red aphid (left) and a green aphid (right) of the same genotype but the latter with *Rickettsiella* infection. (Middle panels) Comparison of body coloration between an original red strain (left), a *Rickettsiella*-injected line that failed to establish infection (center) and an injected line that established *Rickettsiella* infection (right). The hue angle was used as an index of body color. The color bars on the right side approximately indicate the corresponding body color. Different letters (a, b) indicate statistically significant differences ($P < 0.05$, Mann-Whitney U test after Bonferroni correction). (Bottom panels) Relationship between *Rickettsiella* density and aphid body color. *Rickettsiella*-infected insects are indicated in green, while uninfected insects are shown in orange. Each colored number indicates age of an insect (ex. 11 means 11-day-old). Solid lines are regression lines, with statistical significance under the generalized linear model with gamma error.

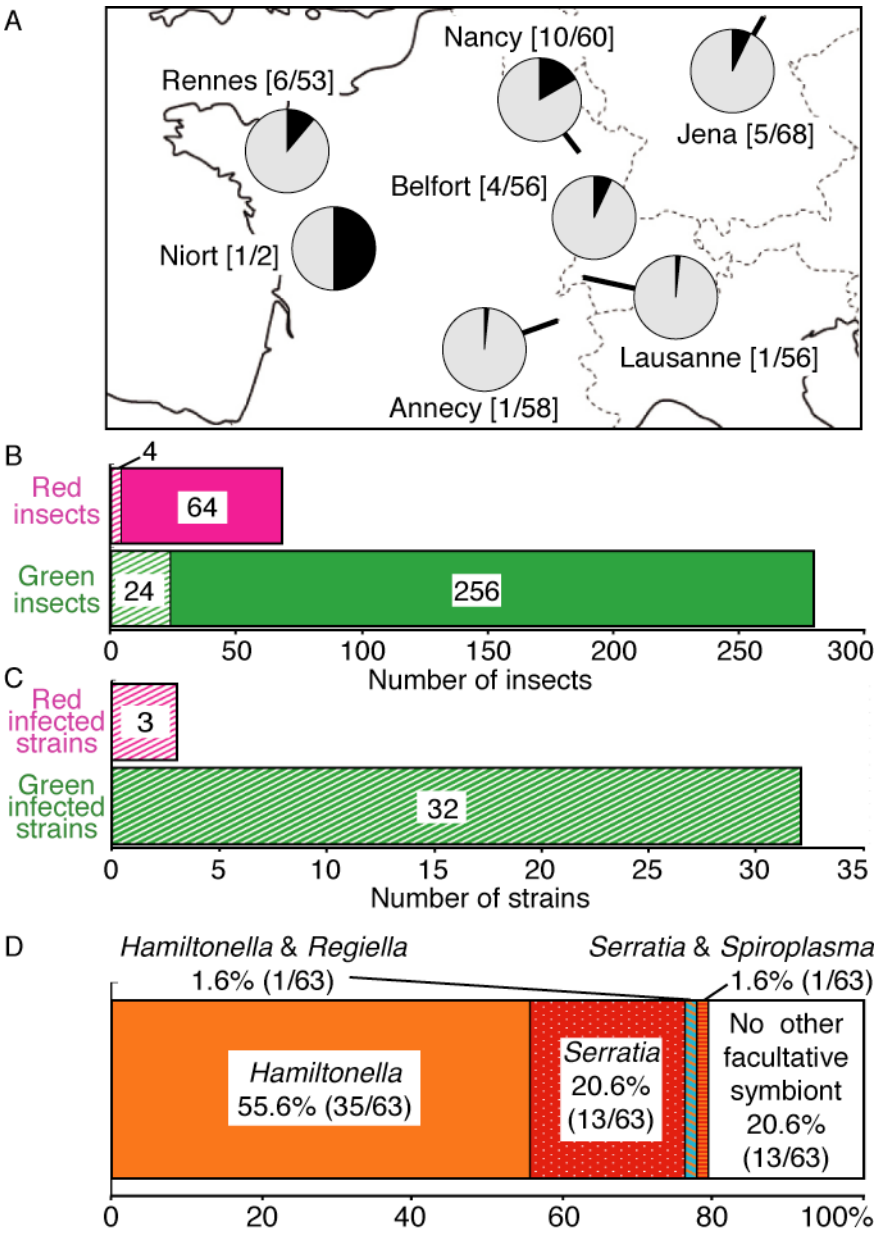

Fig. S2. (A) Diagnostic PCR survey of *Rickettsiella* infection in natural pea aphid populations in Western Europe. Infection frequencies are shown by pie graphs and also by number of infected insects per number of all insects examined in brackets. **(B)** Relationship between body color and *Rickettsiella* infection in 348 pea aphids collected from the European natural populations. (**C**) Body color of 35 laboratory stock strains that were diagnosed as *Rickettsiella*-infected. In (**B**) and (**C**), hatched and filled columns indicate *Rickettsiella*-infected and uninfected, respectively. (**D**) Co-infection of *Rickettsiella* with other facultative symbiont in the pea aphid. In total 63 DNA samples consisting of 28 field-collected insects and 35 laboratory stock strains with *Rickettsiella* infection were subjected to the analysis.

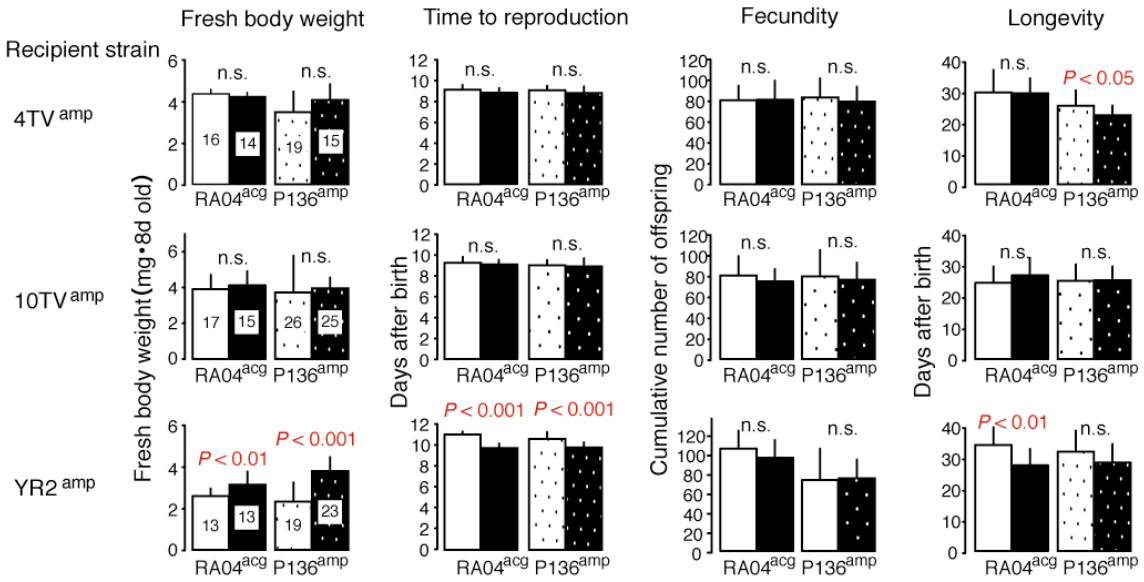

Fig. S3. Fitness effects of *Rickettsiella* infection on transfected aphid strains. *Rickettsiella* derived from two donor strains (RA04^{acg} and P136^{amp}) were introduced into three recipient strains (4TV^{amp}, 10TV^{amp} and YR2^{amp}) (also see table S1). Open bars indicate *Rickettsiella*-uninfected strains, whereas filled bars show *Rickettsiella*-infected strains. Means and standard deviations are shown. Sample sizes are indicated on the columns. Statistically significant differences between *Rickettsiella*-infected and uninfected strains under the same genetic background are shown in red, evaluated under the generalized linear model with appropriate error distribution selected from normal, Poisson, gamma, inverse normal and negative binominal errors according to the Akaike information criterion. n.s. means no significant difference.

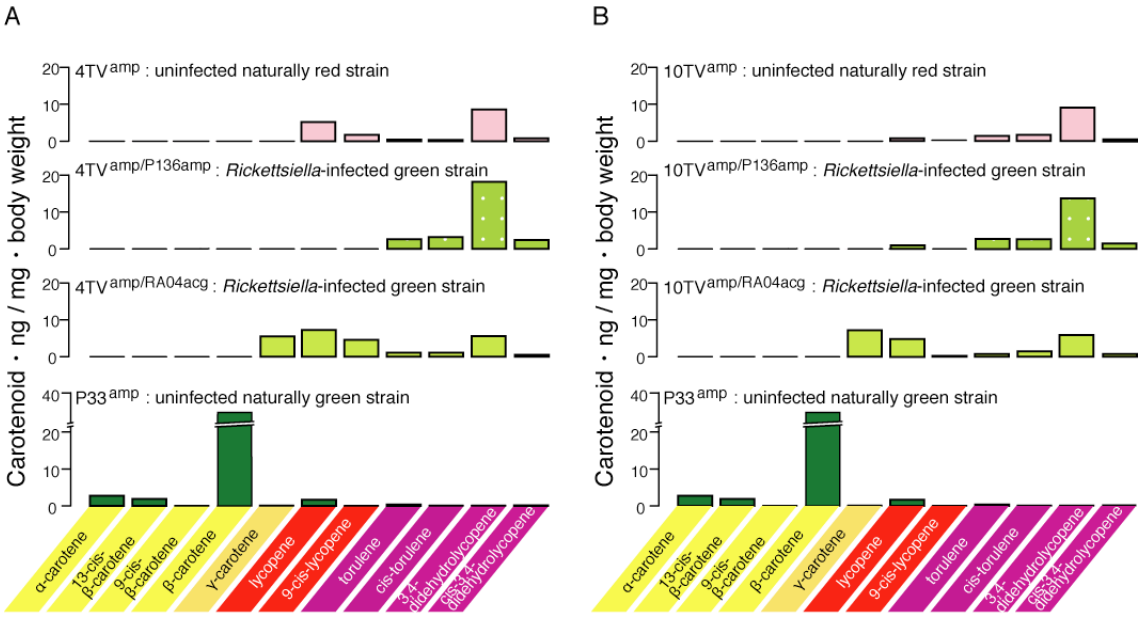

Fig. S4. Carotenoid compositions in uninfected naturally red strains ($4TV^{amp}$, $10TV^{amp}$), *Rickettsiella*-infected green strains ($4TV^{amp/P136amp}$, $4TV^{amp/RA04acg}$, $10TV^{amp/P136amp}$ $10TV^{amp/RA04acg}$), and an uninfected naturally green strain ($P33^{amp}$) of the pea aphid. (A) Comparison under the aphid genetic background $4TV^{amp}$. (B) Comparison under the aphid genetic background $10TV^{amp}$. In both (A) and (B), at the bottom is shown the carotenoid composition of the naturally green strain $P33^{amp}$. The colors highlighting the carotenoid names reflect their colors.

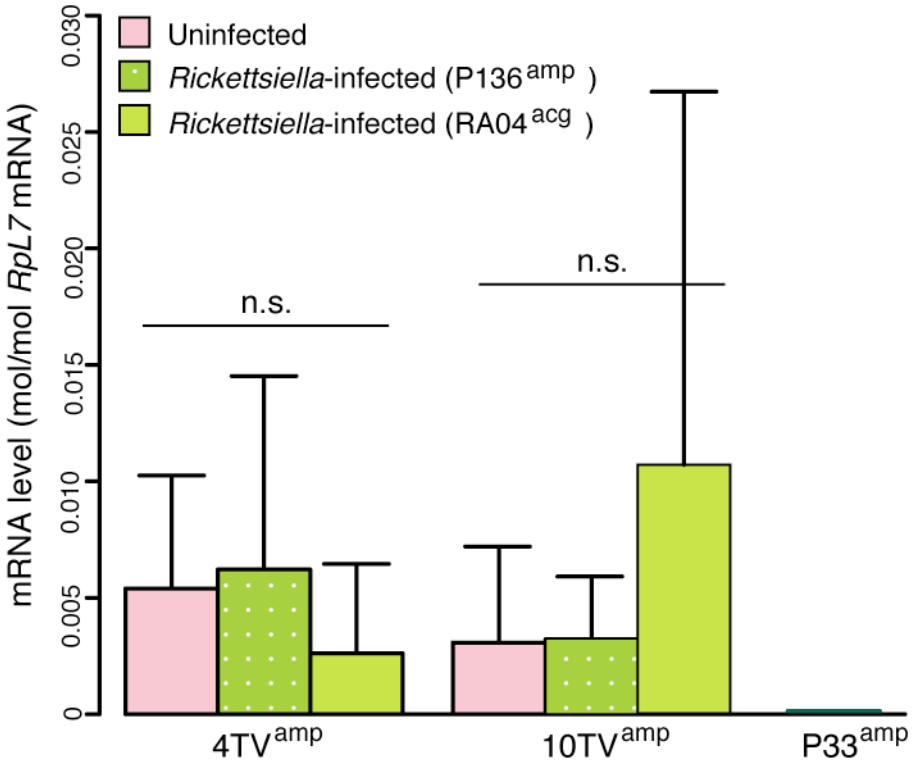

Fig. S5. Expression levels of *tor*, a carotenoid desaturase gene that was laterally transferred from a fungus and is responsible for synthesis of red carotenoids in red pea aphids (*S11*). Columns and bars indicate means and standard errors, respectively (n = 12). The expression levels are shown in terms of mRNA copies of the carotenoid desaturase gene per mRNA copy of *RpL7* gene. The naturally green aphid strain P33 lacking this gene (*S11*) exhibited no expression.

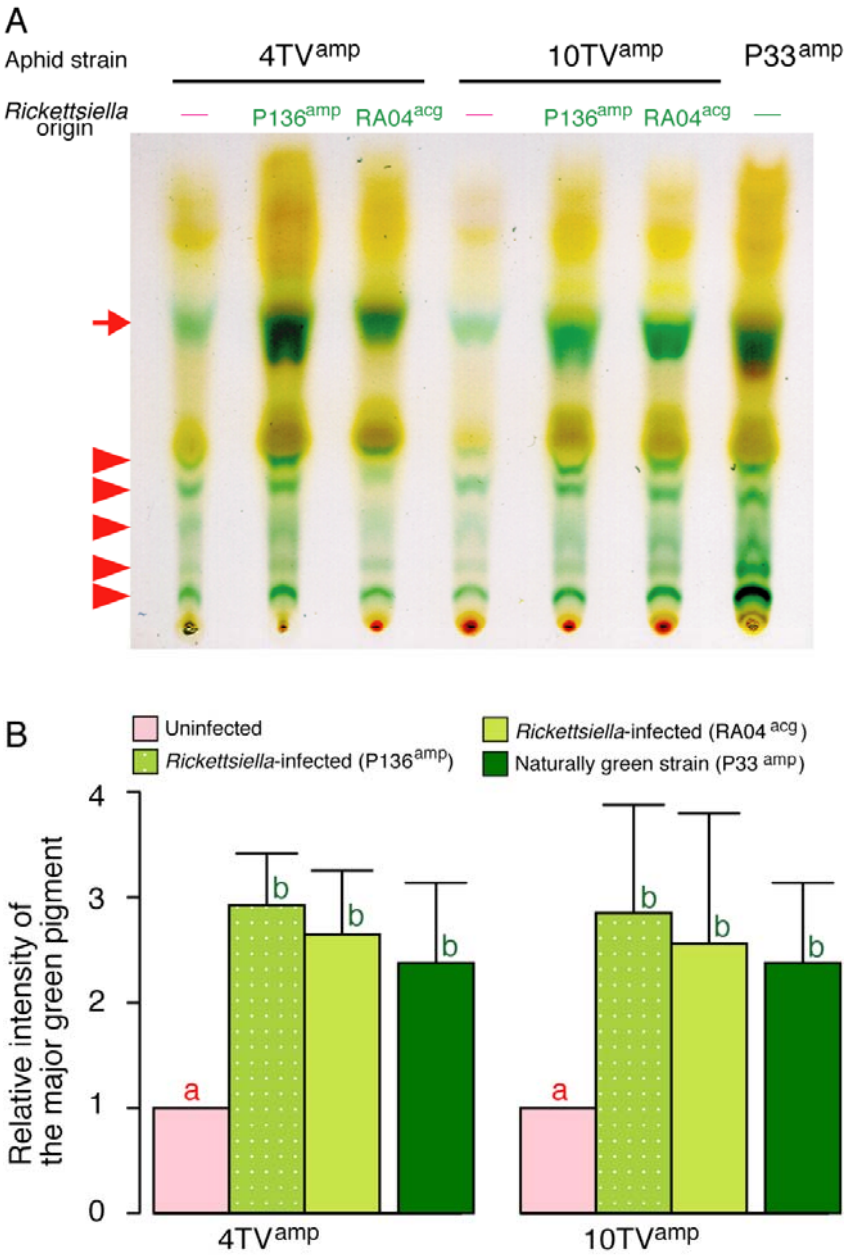

Fig. S6. (**A**) Thin layer chromatography of green pigments from red aphids and *Rickettsiella*-infected green aphids of the same genetic backgrounds. A major green band and five minor ones are shown by an arrow and arrowheads, respectively. Pigment extract on each lane was prepared from the same amount of aphids (8.6 mg wet body weight equivalent). (**B**) Densitometric quantitative comparison of the major green pigment between the red aphids and the *Rickettsiella*-infected green aphids of the same genetic backgrounds. The results are shown as intensity of the green band in the green aphid strains relative to that in the uninfected red aphid strain, either $4TV^{amp}$ or $10TV^{amp}$. Columns and bars indicate means and standard errors, respectively (n=5). Different letters (a and b) indicate statistically significant differences ($P < 0.05$, Mann-Whitney U test after Bonferroni correction).

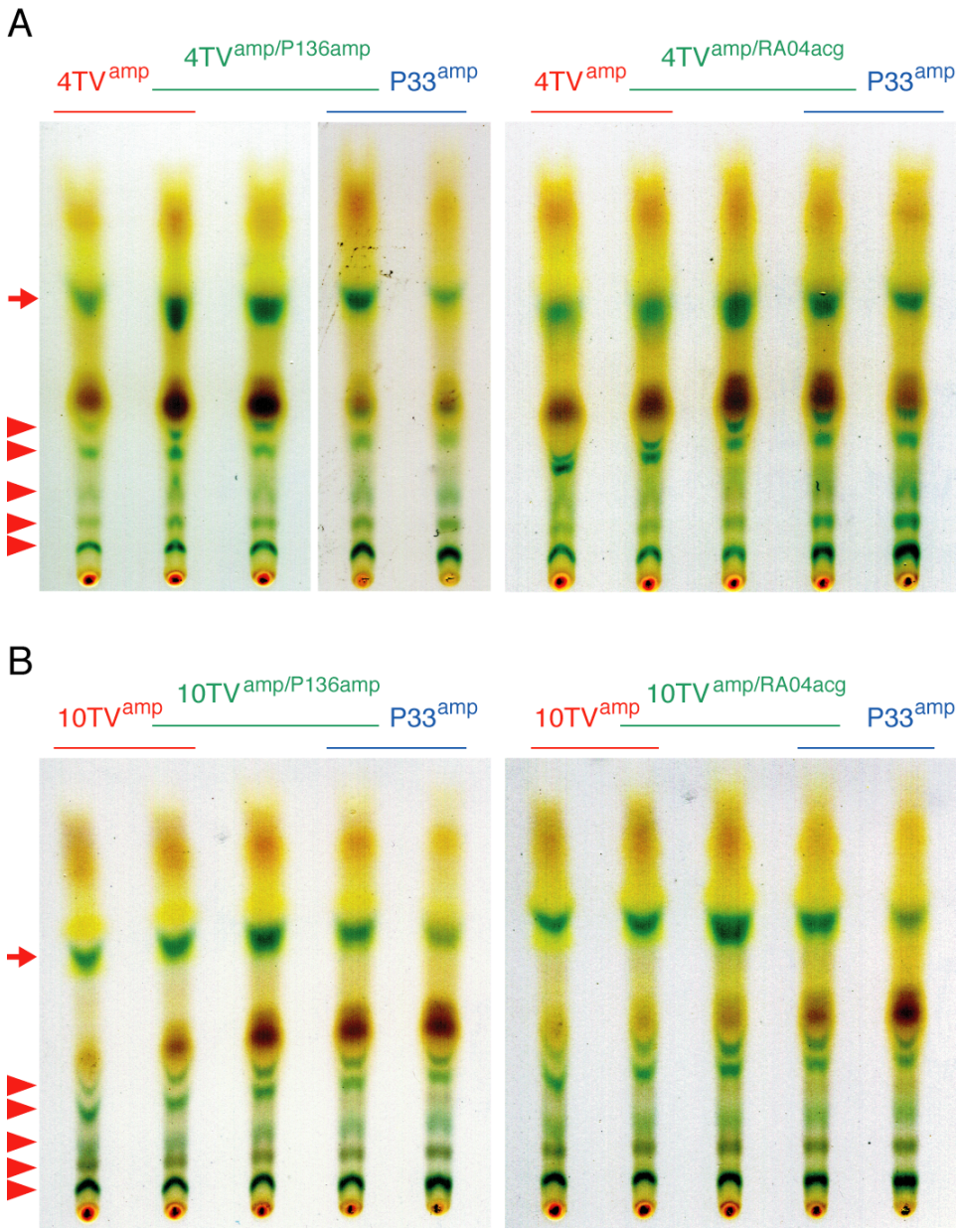

Fig. S7. Co-spotting thin layer chromatography analysis of green pigments from uninfected red aphids and *Rickettsiella*-infected green aphids of the same genetic backgrounds, $4TV^{amp}$ (**A**) and $10TV^{amp}$ (**B**). A major green band and five minor ones are shown by an arrow and arrowheads, respectively. On each lane, roughly the same amount of aphid pigments were spotted and separated. In each overlapping lane, half the amount of the pigments from the left lane was combined with half the amount of the pigment from the right lane. The chromatograms of the overlapping lanes suggest that (i) no new green pigments appear upon *Rickettsiella* infection and (ii) the green pigments in the *Rickettsiella*-infected green aphids are not qualitatively but just quantitatively different from those in the uninfected red aphids.

Table S1 Aphid strains used in this study, their body color, and their *Rickettsiella* infection status.

Aphid strain	Collection locality	Original color	*Rickettsiella* infection (color of adult insects)[a]		
			G1[b]	G2[c]	G3[d]
Strains naturally infected with *Rickettsiella* (co-infecting facultative endosymbiont)[e]					
RA04 (*Hamiltonella*-infected)	Rennes, France	Red -> Green	**16/16 (Green)**	**16/16 (Green)**	**16/16 (Green)**
RA04^{acg} (*Hamiltonella*-eliminated)	Rennes, France	Red -> Green	**16/16 (Green)**	**10/10 (Green)**	**10/10 (Green)**
GCt10 (*Hamiltonella*-infected)	Toulouse, France	Red -> Green	**16/16 (Green)**	**16/16 (Green)**	**16/16 (Green)**
P136 (*Serratia*-infected)	Niort, France	Green	**16/16 (Green)**	**16/16 (Green)**	**16/16 (Green)**
P136^{amp} (*Serratia*-eliminated)	Niort, France	Green	**16/16 (Green)**	**10/10 (Green)**	**10/10 (Green)**
Strains without facultative endosymbiont, artificially injected with *Rickettsiella*[f]					
10TV$^{amp/RA04acg}$	Rennes, France	Red	0/6 (Red) **6/6 (Green)**	0/10 (Red) **10/10 (Green)**	0/10 (Red) **10/10 (Green)**
4TV$^{amp/RA04acg}$	Rennes, France	Red	0/4 (Red) **1/1 (Green)**	0/10 (Red) **10/10 (Green)**	0/10 (Red) **10/10 (Green)**
YR2$^{amp/RA04acg}$	York, UK	Red	0/18 (Red) **4/4 (Green)**	0/10 (Red) **10/10 (Green)**	0/10 (Red) **10/10 (Green)**
10TV$^{amp/P136amp}$	Rennes, France	Red	0/7 (Red) **9/9 (Green)**	0/10 (Red) **10/10 (Green)**	0/10 (Red) **10/10 (Green)**
4TV$^{amp/P136amp}$	Rennes, France	Red	0/7 (Red) **10/10 (Green)**	0/10 (Red) **10/10 (Green)**	0/10 (Red) **10/10 (Green)**
YR2$^{amp/P136amp}$	York, UK	Red	0/12 (Red) **3/3 (Green)**	0/10 (Red) **10/10 (Green)**	0/10 (Red) **10/10 (Green)**
P123$^{amp/P136amp}$	Niort, France	Green	0/7 (Green) **9/9** (Green)	- (Green) **10/10** (Green)	- (Green) **10/10 (Green)**
P33$^{amp/P136amp}$	York, UK	Green	0/5 (Green) **11/11** (Green)	- (Green) **10/10** (Green)	- (Green) **10/10 (Green)**
L100$^{acg/P136amp}$	Rennes, France	Green	0/10 (Green) **6/6** (Green)	- (Green) **10/10** (Green)	- (Green) **10/10 (Green)**
L84$^{acg/P136amp}$	Rennes, France	Green	0/10 (Green) **12/12** (Green)	- (Green) **10/10** (Green)	- (Green) **10/10 (Green)**
L1-22$^{acg/P136amp}$	Rennes, France	Green	0/3 (Green) **5/5** (Green)	- (Green) **10/10** (Green)	- (Green) **10/10 (Green)**
T3-8V1$^{acg/P136amp}$	Rennes, France	Green	0/4 (Green) **4/4** (Green)	- (Green) **10/10** (Green)	- (Green) **10/10 (Green)**
F19$^{amp/P136amp}$	Rennes, France	Green	0/12 (Green) **4/4** (Green)	- (Green) **10/10** (Green)	- (Green) **10/10 (Green)**

[a] Number of infected individuals/number of all individuals examined by diagnostic PCR (body color of 11-day-old adults). Note that both red and green insects appeared in G1 offspring of *Rickettsiella*-injected red insects, and both of them were subjected to diagnostic PCR.

[b] Offspring of *Rickettsiella*-injected adult insects harvested 11 days after injection.

[c] Established from a *Rickettsiella*-uninfected G1 insect and a *Rickettsiella*-infected G1 insect.

[d] Established from a *Rickettsiella*-uninfected G2 insect and a *Rickettsiella*-infected G2 insect.

[e] For example, RA04^{acg} means that the strain RA04 was treated with ampicillin-cefotaxime-gentamycin, whereby *Hamiltonella* infection was eliminated. P136^{amp} means that the strain P136 was treated with ampicillin, whereby *Serratia* infection was eliminated.

[f] For example, 10TV$^{amp/RA04acg}$ means that the strain 10TV^{amp} without facultative endosymbiont was injected with *Rickettsiella*-laden hemolymph from the strain RA04^{acg} .