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# Phenotypic Effect of "*Candidatus* Rickettsiella viridis," a Facultative Symbiont of the Pea Aphid (*Acyrthosiphon pisum*), and Its Interaction with a Coexisting Symbiont

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**A gammaproteobacterial facultative symbiont of the genus *Rickettsiella* was recently identified in the pea aphid, *Acyrthosiphon pisum*. Infection with this symbiont altered the color of the aphid body from red to green, potentially affecting the host's ecological characteristics, such as attractiveness to different natural enemies. In European populations of *A. pisum*, the majority of *Rickettsiella*-infected aphids also harbor another facultative symbiont, of the genus *Hamiltonella*. We investigated this *Rickettsiella* symbiont for its interactions with the coinfecting *Hamiltonella* symbiont, its phenotypic effects on *A. pisum* with and without *Hamiltonella* coinfection, and its infection prevalence in *A. pisum* populations. Histological analyses revealed that coinfecting *Rickettsiella* and *Hamiltonella* exhibited overlapping localizations in secondary bacteriocytes, sheath cells, and hemolymph, while *Rickettsiella*-specific localization was found in oenocytes. *Rickettsiella* infections consistently altered hosts' body color from red to green, where the greenish hue was affected by both host and symbiont genotypes. *Rickettsiella-Hamiltonella* coinfections also changed red aphids to green; this greenish hue tended to be enhanced by *Hamiltonella* coinfection. With different host genotypes, *Rickettsiella* infection exhibited either weakly beneficial or nearly neutral effects on host fitness, whereas *Hamiltonella* infection and *Rickettsiella-Hamiltonella* coinfection had negative effects. Despite considerable frequencies of *Rickettsiella* infection in European and North American *A. pisum* populations, no *Rickettsiella* infection was detected among 1,093 insects collected from 14 sites in Japan. On the basis of these results, we discuss possible mechanisms for the interaction of *Rickettsiella* with other facultative symbionts, their effects on their hosts' phenotypes, and their persistence in natural host populations. We propose the designation "*Candidatus* Rickettsiella viridis" for the symbiont.**

Multiple symbiotic bacteria often coexist within the same host organisms. Many insects are associated with obligately symbiotic bacteria, which are essential for their hosts and exhibit nearly 100% frequencies of infection in these host populations (1, 2). These insects frequently harbor additional, facultatively symbiotic bacteria that are either beneficial, commensal, or parasitic for their hosts, depending on the ecological context, and exhibit partial infection frequencies in host populations (3, 4).

Aphids (Hemiptera: Aphididae), which include more than 5,000 species worldwide (C. Favret and D. C. Eades, Aphid Species File, version 5.0/5.0 [http://aphid.speciesfile.org]), generally have intimate associations with symbiotic microorganisms (5, 6). Among these, the pea aphid (*Acyrthosiphon pisum*) is a model species, for which symbiotic bacteria and their biological functions have been best described (1, 4). In *A. pisum*, and in almost all other aphids, the obligate symbiont *Buchnera aphidicola* is harbored in specialized host cells called bacteriocytes and provides the host with essential amino acids and other nutrients that are deficient in a plant sap diet. Therefore, *Buchnera* is essential for the growth and reproduction of the host and is vertically transmitted through host generations via ovarian passage (1, 5–7). Moreover, *A. pisum* often harbors one or several additional, facultatively symbiotic bacteria, including *Serratia symbiotica*, *Hamiltonella defensa*, *Regiella insecticola*, a *Rickettsia* sp., a *Spiroplasma* sp., and others (8–12). These facultative symbionts are not essential for host growth and reproduction and are subjected mostly to vertical transmission, although they are occasionally transmitted horizontally (4, 13, 14). Notably, some of these facultative symbionts have conditionally beneficial fitness consequences for the host, including resistance to parasitoid wasps or pathogenic fungi (*Hamiltonella*, *Serratia*, or *Regiella*) (15–17), tolerance to heat stress (*Serratia*) (18, 19), or broadening of the food plant range (*Regiella*) (20, 21).

The genus *Rickettsiella* belongs to the order *Legionellales*, which also includes the human-pathogenic genera *Coxiella* and *Legionella*, within the class *Gammaproteobacteria* (22). Members of the genus *Rickettsiella* have been described as pathogens of diverse invertebrates, including insects of the orders Lepidoptera, Orthoptera, Diptera, Dictyoptera, Coleoptera, Hymenoptera, and Hemiptera, in addition to arachnids and crustaceans (23). In infected hosts, *Rickettsiella* bacteria colonize various types of cells and tissues, including the gut, fat body, hemocytes, nerve cells, and oocytes, in which they often have virulent and pathological effects (24–26).

A facultative symbiont belonging to the genus *Rickettsiella* was identified recently in European *A. pisum* populations (12) and

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more recently in *A. pisum* populations in the United Kingdom (27) and North America (28). This aphid-associated *Rickettsiella* species constitutes a lineage phylogenetically distinct from the pathogenic *Rickettsiella* species, such as *Rickettsiella popilliae*, *R. melolonthae*, and *R. grylli*. It is vertically transmitted through host generations via ovarian passage, has no detrimental effects on host fitness, and, strikingly, changes the body color of infected aphids from red to green (12).

This novel *Rickettsiella* symbiont is of great interest because the red/green aphid body color has ecological, evolutionary, and biochemical relevance. In Europe and the United States, red and green aphids commonly coexist within the same populations of *A. pisum*. Previous studies showed that ladybird beetles tended to consume red aphids on green plants (29) and that parasitoid wasps preferentially oviposited into green aphids (29–31), suggesting that these natural enemies with different color preferences may contribute to the color polymorphisms in natural aphid populations. It seems plausible that a color-changing *Rickettsiella* symbiont may affect the evolutionary ecology of a host aphid by modifying prey-predator interactions. In *A. pisum*, a reddish color is caused by carotenoid pigments (32), whereas a greenish color is attributed primarily to polycyclic quinone pigments (12). In our previous study, we showed that *Rickettsiella* infection did not affect the levels of reddish carotenoids but dramatically increased the levels of green polycyclic quinone glycosides, thereby altering a host's body color to greenish, although the mechanisms underlying these processes were unknown (12).

It is noteworthy that a majority of *Rickettsiella*-infected insects in European *A. pisum* populations are coinfected with some other facultative symbiont: 56% with *Hamiltonella* and 21% with *Serratia* (12). It is biologically interesting how these coinfecting symbionts, particularly *Hamiltonella*, influence various features of *Rickettsiella* infection, including effects on the fitness and color phenotypes of the host aphid.

In host-microbe symbiotic associations in general, host genotypes, symbiont genotypes, host-symbiont interactions, and symbiont-symbiont interactions substantially affect the biological parameters of the entire symbiotic system, including a host's fitness and phenotype (33, 34). In this study, we experimentally investigated the interactions between a *Rickettsiella* and a *Hamiltonella* symbiont in *A. pisum* with different combinations of host genotype and symbiont genotype, focusing in particular on the effects on a host's body color and fitness. In addition, we extensively surveyed *A. pisum* populations in Japan for *Rickettsiella* infection. Finally, on the basis of the results obtained in this and previous studies, we have proposed a candidate name for this aphid-associated *Rickettsiella* symbiont.

## MATERIALS AND METHODS

**Insect samples.** Field samples of *A. pisum* were collected from 14 sites across the Japanese archipelago in 2005 (see Table S1 in the supplemental material). At each site, multiple 1-m by 1-m quadrats (at least 1 m apart from each other) were set so as to contain a thick growth of host plants: *Vicia sativa*, *Trifolium repens*, *Trifolium pratense*, or *Medicago sativa*. Considering that aphids reproduce parthenogenetically during the spring and summer, we collected only one wingless adult insect per quadrat to minimize the sampling of identical clones. These aphid samples were immediately preserved in acetone until use (35). Laboratory strains of *A. pisum* with *Rickettsiella* and/or other facultative symbionts were originally collected from France for experimental studies. The symbiont infection status of these strains was either kept as it was originally or experimentally manipulated, as summarized in Table 1. These aphid strains were maintained on *Vicia faba* seedlings at 20°C in a long-day regimen of 16 h of light and 8 h of darkness in the laboratories of T. Tsuchida and T. Fukatsu in Japan with the permission of the Nagoya and Yokohama Plant Protection Stations, respectively.

**DNA analysis.** DNA was extracted from individual aphid samples by using conventional proteinase K digestion, phenol extraction, and ethanol precipitation. Each purified DNA sample was dissolved in 500 μl of TE buffer (10 mM Tris-HCl [pH 8.0] and 0.1 mM EDTA). Bacterial 16S rRNA genes of the symbionts were amplified by PCR with *Ex Taq* DNA polymerase (TaKaRa, Tokyo, Japan) using the forward primer 16SA1 in combination with the reverse primer 16SB1 or γ940R (see Table S2 in the supplemental material). PCR products were subjected to cloning, restriction fragment length polymorphism genotyping, and DNA sequencing, as described previously (36). Diagnostic PCR detection of specific symbionts was performed using HybriPol DNA polymerase (Bioline, London, United Kingdom) and the buffer system supplied, with primers specific for the respective symbionts (listed in Table S2). The PCR temperature profile was 95°C for 3 min, followed by 35 cycles of 95°C for 30 s and 55°C for 30 s, and a final extension at 72°C for 1 min. Real-time PCR detection of *Rickettsiella* was performed on a MX3005P real-time PCR system (Agilent Technologies) with specific primers (listed in Table S2) by using a standard curve method as described previously (37). The PCR conditions were the same as those for the diagnostic PCR analysis except that 40 cycles were used. To avoid misdiagnosis, we considered the samples of interest to be *Rickettsiella* positive only if they met all of the following criteria: (i) the melting temperature ($T_m$) of each of the amplicons was virtually identical to that of the positive control; (ii) the amplification efficiency was higher than that of the negative control; (iii) the gene copy numbers were >10 per PCR tube, which is equivalent to 1,000 copies per insect; and (iv) two different primer sets produced specific amplicons. Total-DNA preparations from *A. pisum* strains whose facultative symbionts had been definitively identified were used as control DNA samples for both diagnostic and real-time PCR analyses: strain RA04$^{acg}$ with *Rickettsiella* (12), strain L100 with *Hamiltonella* (38), strain TUt with *Regiella* (39), and strain AIST with no facultative symbionts (36).

***In situ* hybridization.** *Buchnera*, *Rickettsiella*, and *Hamiltonella* were visualized in *A. pisum* embryos of strain GCt10 (Table 1) by whole-mount fluorescence *in situ* hybridization (FISH), as described previously (40), using the fluorochrome-labeled oligonucleotide probes listed in Table S2 in the supplemental material. Host cell nuclei were counterstained with 4′,6-diamino-2-phenylindole. Observations were made using a laser scanning confocal microscope (LSM 5 Pascal; Carl Zeiss). The types of cells to which the symbionts were localized were differentiated by their morphological characteristics as reported in previous studies (12, 41, 42). Pseudocolors were assigned to display *Buchnera*, *Rickettsiella*, *Hamiltonella*, and host nuclear DNA in the same images. Combinations of two symbionts each were analyzed in series because all symbionts and host nuclei could not be detected simultaneously with the three-laser unit system used in this study. The specificity of *in situ* hybridization was confirmed by the following control experiments: a no-probe control, an RNase digestion control, and a competitive suppression control with excessive unlabeled probe (43).

**Establishment of symbiont-manipulated *A. pisum* strains.** *Hamiltonella*, *Serratia*, *or Regiella* infection was selectively eliminated from *A. pisum* strains by antibiotic treatments, without affecting *Buchnera* or *Rickettsiella* infections, as described previously (12, 44). Experimental transfection of *Hamiltonella*, *Serratia*, *Regiella*, or *Rickettsiella* was conducted using hemolymph injection as described previously (41). Newborn nymphs deposited by injected mothers were collected at 11 days after injection because almost 100% infection with the facultative symbionts was reported from that day onward (8, 42). The collected nymphs were reared individually, and isofemale lines were established from them. Symbiont infections in these isofemale lines were confirmed by diagnostic

**TABLE 1** Aphid strains used in this study

| Strain | Original locality/original strain and treatment | Infection status with facultative symbionts[a] (sequence accession no.)[b] | Expt(s)[c] | Source or reference(s) |
|---|---|---|---|---|
| 4TV | Collected at Rennes, France | *Regiella* | | 38 |
| 10TV | Collected at Rennes, France | *Regiella* | | 38 |
| TUt | Collected at Tsuchiura, Japan | *Regiella* (**AB780460**) | | 20, 21, 39, 42 |
| GCt10 | Collected at Toulouse, France | *Rickettsiella* (AB522703), *Hamiltonella* (**AB780461**) | ISH | 12 |
| RA04 | Collected at Rennes, France | *Rickettsiella* (AB522705), *Hamiltonella* (**AB780462**) | | 12 |
| RA04$^{acg}$ | RA04, treated with ampicillin, cefotaxime, and gentamicin[d] | *Rickettsiella* | | 12 |
| P136 | Collected at Niort, France | *Rickettsiella* (AB522702), *Serratia* (**AB780463**) | | 12 |
| P136$^{amp}$ | P136, treated with ampicillin[e] | *Rickettsiella* | | 12 |
| CLs07 | Collected at Castelnaudary, France | *Rickettsiella* (**AB780464**), *Hamiltonella* (**AB780465**) | | This study |
| GCe21 | Collected at Gers, France | *Rickettsiella* (**AB780466**), *Hamiltonella* (**AB780467**) | | This study |
| GCe50 | Collected at Gers, France | *Rickettsiella* (**AB780468**), *Hamiltonella* (**AB780469**) | | This study |
| 4TV$^{amp}$ | 4TV, treated with ampicillin | Uninfected | Fitness, color | 12 |
| 4TV$^{amp/TUt}$ | 4TV$^{amp}$, injected with hemolymph of TUt | *Regiella* | Color | This study |
| 4TV$^{amp/GCt10(R)}$ | 4TV$^{amp}$, injected with hemolymph of GCt10 | *Rickettsiella*[f] | Fitness, color | This study |
| 4TV$^{amp/GCt10(H)}$ | 4TV$^{amp}$, injected with hemolymph of GCt10 | *Hamiltonella*[f] | Fitness, color | This study |
| 4TV$^{amp/RA04acg}$ | 4TV$^{amp}$, injected with hemolymph of RA04$^{acg}$ | *Rickettsiella* | Color | 12 |
| 4TV$^{amp/P136amp}$ | 4TV$^{amp}$, injected with hemolymph of P136$^{amp}$ | *Rickettsiella* | Color | 12 |
| 4TV$^{amp/GCt10}$ | 4TV$^{amp}$, injected with hemolymph of GCt10 | *Rickettsiella*, *Hamiltonella* | Fitness, color | This study |
| 4TV$^{amp/RA04}$ | 4TV$^{amp}$, injected with hemolymph of RA04 | *Rickettsiella*, *Hamiltonella* | Color | This study |
| 4TV$^{amp/CLs07}$ | 4TV$^{amp}$, injected with hemolymph of CLs07 | *Rickettsiella*, *Hamiltonella* | Color | This study |
| 4TV$^{amp/GCe21}$ | 4TV$^{amp}$, injected with hemolymph of GCe21 | *Rickettsiella*, *Hamiltonella* | Color | This study |
| 4TV$^{amp/GCe50}$ | 4TV$^{amp}$, injected with hemolymph of GCe50 | *Rickettsiella*, *Hamiltonella* | Color | This study |
| 4TV$^{amp/P136}$ | 4TV$^{amp}$, injected with hemolymph of P136 | *Rickettsiella*, *Serratia* | Color | 38 |
| 10TV$^{amp}$ | 10TV, treated with ampicillin | Uninfected | Fitness, color | 12 |
| 10TV$^{amp/RA04acg}$ | 10TV$^{amp}$, injected with hemolymph of RA04$^{acg}$ | *Rickettsiella* | Fitness, color | 12 |
| 10TV$^{amp/RA04(H)}$ | 10TV$^{amp}$, injected with hemolymph of RA04 | *Hamiltonella*[f] | Fitness, color | This study |
| 10TV$^{amp/P136amp}$ | 10TV$^{amp}$, injected with hemolymph of P136$^{amp}$ | *Rickettsiella* | Color | This study |
| 10TV$^{amp/RA04}$ | 10TV$^{amp}$, injected with hemolymph of RA04 | *Rickettsiella*, *Hamiltonella* | Fitness, color | This study |
| 10TV$^{amp/P136}$ | 10TV$^{amp}$, injected with hemolymph of P136 | *Rickettsiella*, *Serratia* | Color | 38 |

[a] The infection status was confirmed by diagnostic PCR. All strains were infected with the essential symbiont *Buchnera*. "Uninfected" means that the strain was infected with *Buchnera* but not with any facultative symbionts.
[b] Accession numbers for sequences obtained in this study are shown in boldface.
[c] Strains were used for the following experiments: *in situ* hybridization (ISH), measurement of fitness, and evaluation of color-changing effects.
[d] The mixture of antibiotics, each at a dose of 250 μg/ml, is described in reference 12.
[e] Selective elimination of *Serratia* was performed by use of ampicillin as described in references 41 and 44.
[f] The single infection was fortuitously obtained by injecting both *Rickettsiella*- and *Hamiltonella*-infected hemolymph.

PCR for at least three successive generations. In this way, we generated *A. pisum* strains with controlled genetic backgrounds that were infected with different combinations of facultative symbionts: *Rickettsiella*, *Hamiltonella*, *Serratia*, and *Regiella* (Table 1).

**Body color measurements.** Body color measurements were taken for 30 adult insects (11 days old) of each aphid strain as described previously (12) with minor modifications. Insects were anesthetized with carbon dioxide and were placed on a filter paper. Their color images were acquired with a digital camera (DFC295; Leica) attached to a microscope (M165C; Leica). These images were analyzed using Adobe Photoshop CS5 extended software (version 12.0). Ten points (11 pixels by 11 pixels each) were randomly chosen from the abdominal region of each insect, and three color factors, hue, brightness, and saturation (see Fig. S1 in the supplemental material), were acquired from each point. Mean hue, saturation, and brightness values were calculated for each insect and were defined as the body color indices of the insect. These values were statistically analyzed by the Wilcoxon rank-sum test with Bonferroni's correction.

**Fitness measurements.** Adult insects at 2 days after emergence were allowed to produce nymphs for 6 h on broad bean plants. After removal of the adult insects, the newborn nymphs were kept on the same plants for 3 days. Subsequently, each newborn nymph was individually placed on a 1-week-old broad bean seedling and was reared at 20°C under a long-day regimen of 16 h of light and 8 h of darkness. The plant was renewed every week. For each insect, the fresh body weight at 8 days after birth, the time to first reproduction, the total number of offspring, and longevity were recorded.

**Nucleotide sequence accession numbers.** The nucleotide sequences determined in this study were deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB780460 to AB780469.

## RESULTS

***In vivo* localization of *Rickettsiella* and coexisting *Hamiltonella*.** Figure 1 shows the *in vivo* localizations of *Rickettsiella* and *Hamiltonella* in a naturally coinfected *A. pisum* strain, GCt10, as visualized by whole-mount fluorescence *in situ* hybridization of dissected embryos. *Rickettsiella* was preferentially detected in secondary bacteriocytes, in sheath cells constituting the bacteriomes, and in oenocytes scattered within the body cavity (Fig. 1A). *Hamiltonella* was also localized in secondary bacteriocytes and sheath cells, but not in oenocytes (Fig. 1B). The infection patterns of *Rickettsiella* and *Hamiltonella* appeared spatially sporadic; some secondary bacteriocytes and sheath cells were coinfected with both symbionts, whereas other secondary bacteriocytes and sheath cells were infected with only one of these symbionts (Fig. 1C). *Buchnera* was consistently found in primary bacteriocytes (Fig. 1A and B).

**Effects *of Rickettsiella* and coexisting facultative symbionts on host body color in the 4TV$^{amp}$ genetic background.** Figure 2 and Fig. S2A in the supplemental material demonstrate the effects of facultative symbiont infections on aphid body color in the 4TV$^{amp}$ genetic background. *Rickettsiella*-infected and *Rickettsiella-Hamiltonella*-coinfected insects appeared greenish in color in contrast to reddish uninfected insects, whereas *Regiella*-infected

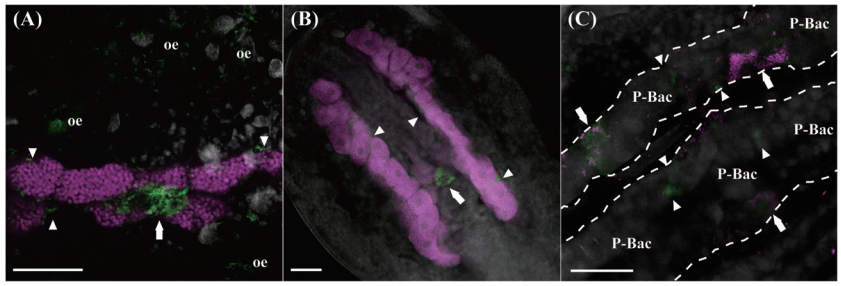


**FIG 1** FISH analysis of aphid embryos from strain GCt10 infected with three symbiotic bacteria. (A) Primary bacteriocytes containing *Buchnera* (magenta) and secondary bacteriocytes, a sheath cell, and oenocytes harboring *Rickettsiella* (green). (B) *Buchnera* (magenta) and *Hamiltonella* (green). (C) *Rickettsiella* (magenta) and *Hamiltonella* (green) are both within secondary bacteriocytes and sheath cells. White dashed lines, outlines of bacteriomes; arrows, secondary bacteriocytes; arrowheads, sheath cells; oe, oenocytes; P-Bac, primary bacteriocytes. Bars, 50 μm.

and *Hamiltonella*-infected insects appeared similar to uninfected insects (see Fig. S2A). Quantitative color measurements confirmed this visual impression.

*Rickettsiella*-infected, *Rickettsiella-Hamiltonella*-coinfected, and *Rickettsiella-Serratia*-coinfected insects exhibited significantly more greenish hues (i.e., higher hue values [see Fig. S1 in the supplemental material]) and lower brightness values than uninfected, *Regiella*-infected, and *Hamiltonella*-infected insects (Fig. 2A and C). Most *Rickettsiella*-infected, *Rickettsiella-Hamiltonella*-coinfected, and *Rickettsiella-Serratia*-coinfected insects exhibited significantly higher saturation values than uninfected insects (Fig. 2B). In contrast, *Regiella*-infected and *Hamiltonella*-infected insects showed no significant differences from uninfected insects (Fig. 2A to C).

Colorimetric analysis also highlighted other subtle color differences. For example, different strains of *Rickettsiella* symbionts caused significantly different body color brightness levels (Fig. 2C). *Rickettsiella-Hamiltonella*-coinfected insects tended to have a little more greenish hue than *Rickettsiella*-infected insects. In particular, the greenish hue levels of *Rickettsiella*-infected strain $4TV^{amp/GCt10(R)}$ and *Rickettsiella-Hamiltonella*-coinfected strain $4TV^{amp/GCt10}$ were significantly different, although these strains had identical *Rickettsiella* genotypes (Fig. 2A).

**Effects *of Rickettsiella* and coexisting facultative symbionts on host body color in the $10TV^{amp}$ genetic background.** Figure 3 and Fig. S2B in the supplemental material demonstrate the effects of facultative symbiont infections on aphid body color in the $10TV^{amp}$ genetic background. As with $4TV^{amp}$ insects, *Rickettsiella*-infected and *Rickettsiella-Hamiltonella*-coinfected insects were more greenish in color than uninfected and *Hamiltonella*-infected insects (see Fig. S2B). Color measurements objectively supported these observations.

*Rickettsiella*-infected, *Rickettsiella-Hamiltonella*-coinfected, and *Rickettsiella-Serratia*-coinfected insects had significantly higher greenish hue values and lower brightness values than uninfected and *Hamiltonella*-infected insects (Fig. 3A and C). In addition, *Rickettsiella*-infected, *Rickettsiella-Hamiltonella*-coinfected, and *Rickettsiella-Serratia*-coinfected insects had significantly higher saturation values than uninfected insects (Fig. 3B). In contrast to the changes in $4TV^{amp}$ insects, *Hamiltonella* infection changed $10TV^{amp}$ insects' body color from yellow-reddish (for uninfected insects) to yellow-greenish, which appeared to be due to a reduction in red color (see Fig. S2B in the supplemental material). Quantitative color measurements confirmed this visual impression. *Hamiltonella*-infected insects had significantly increased greenish hue, higher saturation, and higher brightness values than uninfected insects (Fig. 3A to C).

This colorimetric analysis also highlighted other subtle color differences. For example, greenish hue levels were significantly different for two *Rickettsiella*-infected insect strains, despite their identical host genotypes (Fig. 3A). The *Rickettsiella-Hamiltonella*-coinfected strain $10TV^{amp/RA04}$ had a significantly higher greenish hue level than *Rickettsiella*-infected strain $10TV^{amp/RA04acg}$, despite the identical *Rickettsiella* genotypes (Fig. 3A).

**Effects of *Rickettsiella* and coexisting facultative symbionts on host fitness parameters in the $4TV^{amp}$ genetic background.** In the $4TV^{amp}$ genetic background, *Rickettsiella*-infected insects showed no significant differences in fitness from uninfected insects (Fig. 4A to D), whereas *Hamiltonella*-infected insects were significantly smaller and reproduced significantly more slowly than uninfected insects (Fig. 4A and B). The fitness values of *Rickettsiella-Hamiltonella*-coinfected insects tended to be negatively affected compared with those of *Rickettsiella*-infected insects and uninfected insects: their body weight was lower than that of uninfected insects; their time to reproduction was longer than that of *Rickettsiella*-infected insects; and they had fewer offspring than did *Rickettsiella*-infected insects (Fig. 4A to C). However, longevity showed no significant differences (Fig. 4D). No significant differences in fitness were found between *Hamiltonella*-infected insects and *Rickettsiella-Hamiltonella*-coinfected insects (Fig. 4A to D).

**Effects of *Rickettsiella* and coexisting facultative symbionts on host fitness parameters in the $10TV^{amp}$ genetic background.** In the $10TV^{amp}$ genetic background, *Rickettsiella*-infected insects notably had significantly greater body weights and a shorter time to reproduction than uninfected insects (Fig. 4E and F). In contrast, *Hamiltonella*-infected insects produced significantly fewer offspring than uninfected and *Rickettsiella*-infected insects (Fig. 4G). The fitness values of *Rickettsiella-Hamiltonella*-coinfected insects were generally similar to those of *Hamiltonella*-infected insects (Fig. 4E to H).

**Infection prevalence of *Rickettsiella* in Japanese *A. pisum* populations.** We examined *A. pisum* individuals that were collected at 14 sites across the Japanese archipelago for *Rickettsiella* infection (see Table S1 in the supplemental material). Diagnostic PCR using two sets of specific primers that targeted the *Rickettsiella* 16S rRNA gene and *gyrB* gene consistently detected no *Rickettsiella* infection in 1,093 insects examined. Highly sensitive real-

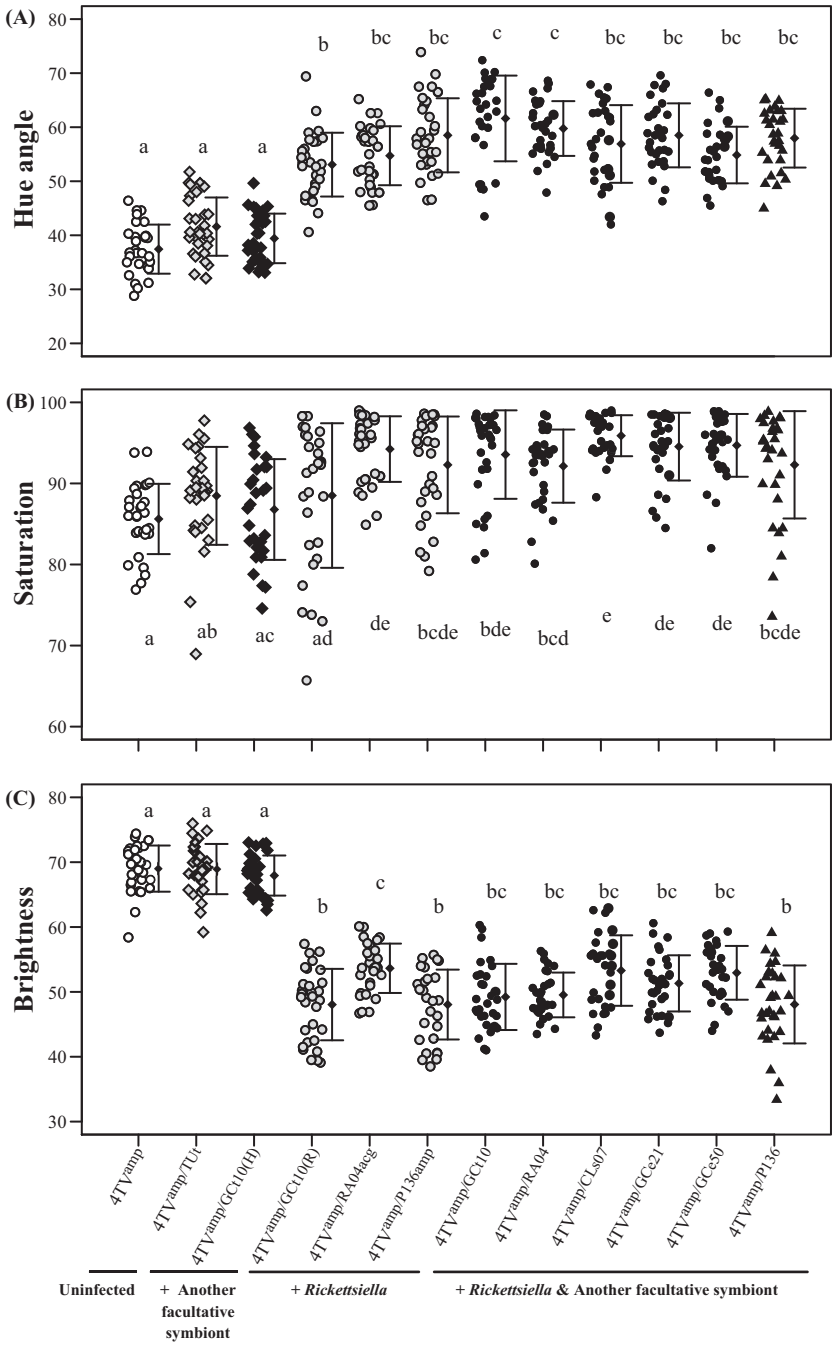


**FIG 2** Comparison of body color between the original red strain 4TV$^{amp}$ (open circles), a strain infected with *Regiella* (shaded diamonds), a strain infected with *Hamiltonella* (filled diamonds), strains infected with *Rickettsiella* (shaded circles), strains infected with *Rickettsiella* and *Hamiltonella* (filled circles), and a strain infected with *Rickettsiella* and *Serratia* (filled triangles). Thirty insects were used per strain. The strains used in this experiment are listed in Table 1. Different lowercase letters indicate statistically significant differences ($P$, $<0.05$ by the Wilcoxon rank-sum test with Bonferroni's correction).

time PCR using the same primer sets also detected no *Rickettsiella* infection in 106 randomly chosen insects.

## DISCUSSION

***In vivo* localization of the *Rickettsiella* symbiont and a coexisting facultative symbiont.** Previously reported bacteria belonging to the genus *Rickettsiella* were mostly perceived as invertebrate pathogens, which typically multiplied within acidic vacuoles in various host tissues (23). In contrast, the *Rickettsiella* symbiont of *A. pisum* was densely accumulated within bacteriocytes and sheath cells (Fig. 1A), at least at the embryonic stages, although it was also observed in other tissues, including oenocytes (Fig. 1A), ovariole pedicels, and a posterior part of the ovary, as reported in a previous study (12). The *Rickettsiella* symbiont is also present in hemolymph on account of successful transfection via hemolymph injection (Table 1) (12). Note that other facultative symbionts of

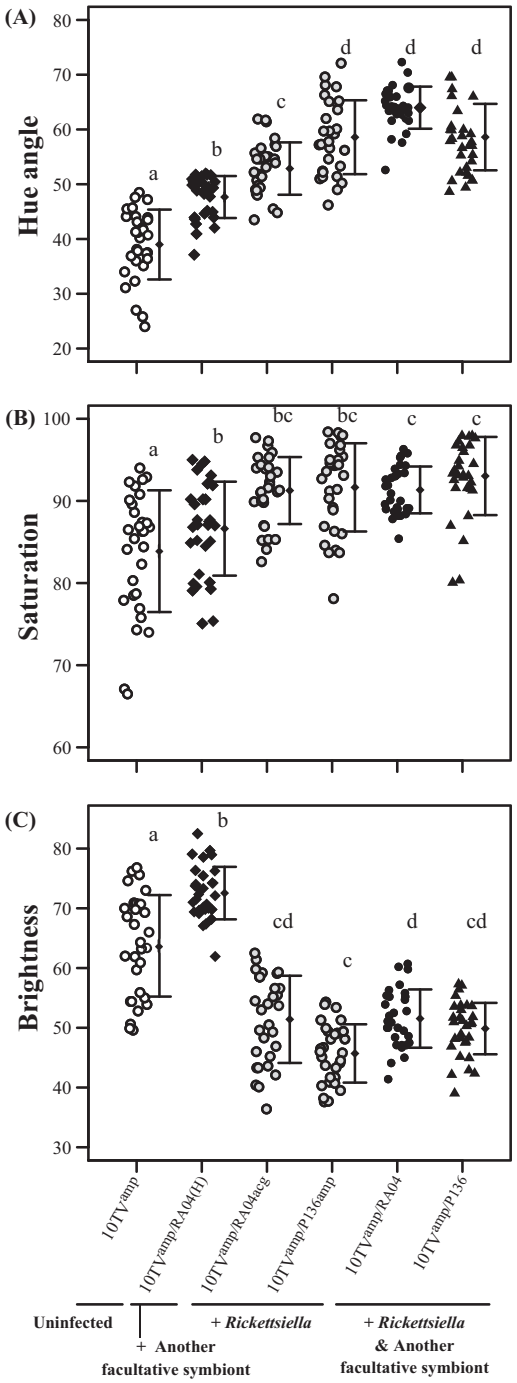


**FIG 3** Comparisons of body color between the original red strain 10TV$^{amp}$ (open circles), a strain infected with *Hamiltonella* (filled diamonds), strains infected with *Rickettsiella* (shaded circles), a strain infected with *Rickettsiella* and *Hamiltonella* (filled circles), and a strain infected with *Rickettsiella* and *Serratia* (filled triangles). Thirty insects were used per strain. The strains used in this experiment are listed in Table 1. Different lowercase letters indicate statistically significant differences ($P$, <0.05 by the Wilcoxon rank-sum test with Bonferroni's correction).

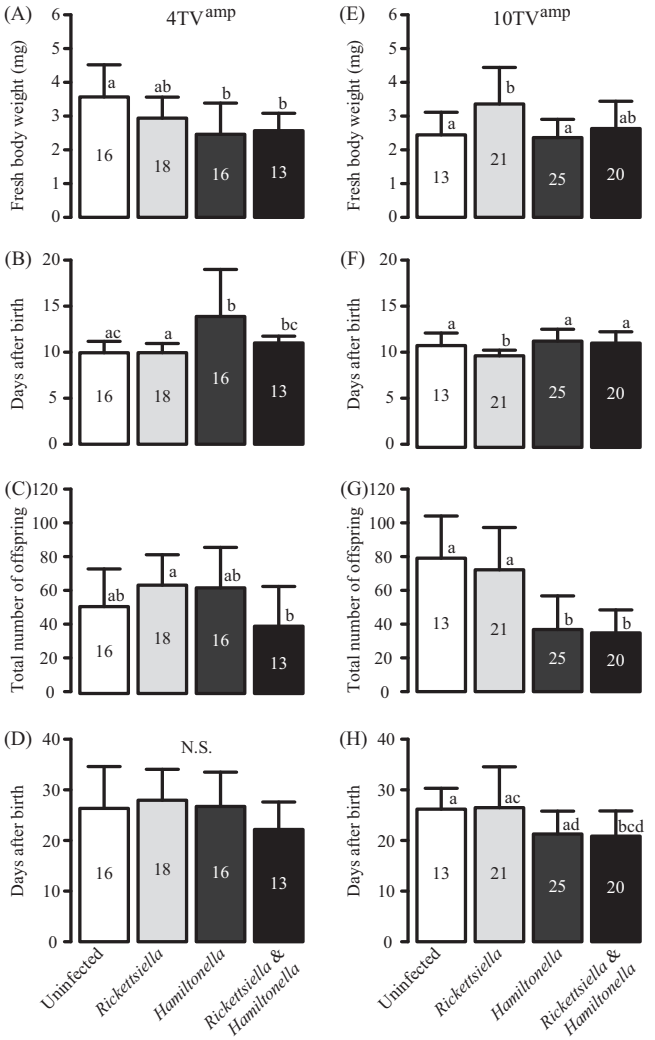


**FIG 4** Fitness effects of infection with either *Rickettsiella*, *Hamiltonella*, or both on two aphid strains, 4TV$^{amp}$ (left) and 10TV$^{amp}$ (right). The strains used in this experiment are listed in Table 1. (A and E) Fresh body weight (mg) of 8-day-old aphids; (B and F) time to reproduction; (C and G) total number of offspring; (D and H) longevity. Results are means and standard deviations. The number of insects used is given in each bar. Different lowercase letters indicate statistically significant differences ($P$, <0.05 by the Wilcoxon rank-sum test with Bonferroni's correction). N.S., not significant.

*A. pisum* have also been detected in bacteriocytes, sheath cells, and hemolymph (9, 10, 36, 41, 42, 45). On the other hand, no localization to oenocytes, ovariole pedicels, or the posterior ovary has been reported for the other facultative symbionts. These observations suggest that (i) the *Rickettsiella* symbiont probably has some molecular and cellular mechanisms for its infection and maintenance in specific host cells and tissues which are common to those operating in the other facultative symbionts, (ii) the mechanisms, meanwhile, are somewhat different from those of the other facultative symbionts, which may be relevant to the *Rickettsiella*-specific cellular tropism to, for example, oenocytes, and (iii) the color-changing effects of the *Rickettsiella* symbiont may be relevant to its unique cellular tropism, although this is speculative.

Therefore, *Rickettsiella* and *Hamiltonella* often coexist in the same bacteriocytes and sheath cells (Fig. 1C) and also in hemolymph, sharing the same ecological niches within the host body, which must facilitate various biological interactions between the coexisting symbionts and influence their effects on the host insect phenotype.

**Influence of the differences among *Rickettsiella* strains, *Hamiltonella* strains, and a combination of *Rickettsiella* and *Hamiltonella* strains on the host body color.** We examined the color-changing effects of several *Rickettsiella* strains in identical host genetic backgrounds. Body colors differed significantly depending on the *Rickettsiella* strain (Fig. 2 and 3; see also Fig. S2 in the supplemental material). One causal factor for the differences between these *Rickettsiella* strains may be differences in the genetic elements that are directly related to body color changes. Even if they are identified as belonging to the same *Rickettsiella* species, different bacterial strains are sometimes vastly different, genetically and phenotypically, from each other. For example, the aphid symbiont *Regiella insecticola* strain 5.15 confers protection against parasitoids, whereas *R. insecticola* strain LSR1 has no protective activity, probably because of some differences in their genomes (46). These differences among *Rickettsiella* strains may account for their different effects on the host's body color. Alternatively, the different *Rickettsiella* strains may differentially affect the physiological status of the host aphids. This could indirectly affect the body colors of aphids.

When the aphid strain 10TV$^{amp}$ was infected with *Hamiltonella* derived from the aphid strain RA04, its body color was significantly changed (Fig. 3; see also Fig. S2B in the supplemental material). This color change appeared to be caused by a reduction in the amounts of some red pigments (see Fig. S2B), which is likely due to severe detrimental effects of the *Hamiltonella* infection (Fig. 4E to H). This explanation is supported by the fact that the aphid strain 4TV$^{amp}$ infected with another *Hamiltonella* strain showed only mild symptoms (Fig. 4A to D) and did not exhibit any significant body color change [4TV$^{amp/GCt10(H)}$] (Fig. 2). Regardless of its fitness effects, *Hamiltonella* affected the host's body color slightly, making it more greenish, in the context of *Hamiltonella-Rickettsiella* coinfection (Fig. 2 and 3; see also Fig. S2), suggesting the possibility that *Hamiltonella* may somehow affect the biological functions of *Rickettsiella*, the physiology of the host aphid, or both.

The molecular mechanisms underlying *Rickettsiella*-induced body color changes are currently unknown, although the requisite pigments appear to be produced by the host aphid (12). These mechanisms could be determined by comparing the biomolecules of the strains of aphids whose body colors differed significantly with different infections, as demonstrated in this study.

**Fitness effects of *Rickettsiella*, *Hamiltonella*, and *Rickettsiella-Hamiltonella* infections.** We examined the effects of infections with *Rickettsiella*, *Hamiltonella*, or both on the fitness components of *A. pisum* (Fig. 4). In contrast to the findings for other, pathogenic *Rickettsiella* species, no detrimental effects were detected for aphids infected with *Rickettsiella* symbionts. Rather, the *Rickettsiella* infections had weakly beneficial or almost neutral effects on the host, depending on the combination of host and symbiont genotypes (Fig. 4). These results were in agreement with those of a previous study (12). In contrast, negative fitness effects were observed for *Hamiltonella* infections, as reported previously (38, 47). Detrimental effects were also observed for those aphids that were coinfected with *Rickettsiella* and *Hamiltonella*. These fitness costs could be attributed to *Hamiltonella* infection, because the tendency toward negative effects was similar to that for infection with *Hamiltonella* alone.

**Possible mechanisms for maintenance of *Rickettsiella-Hamiltonella* coinfection in *A. pisum* populations.** The negative fitness effects of *Rickettsiella-Hamiltonella* coinfection could lead to decreases in the frequencies of these symbionts in natural populations. Nevertheless, despite the negative effects, *Rickettsiella-Hamiltonella*-coinfected insects are found at considerable frequencies in Western European (12) and North American (28) populations of *A. pisum*. These observations suggest that there may be some mechanisms for generating and maintaining multiple infections of *Rickettsiella* and *Hamiltonella* in *A. pisum* populations. If we assume an initial population with only single *Rickettsiella* or *Hamiltonella* infections, horizontal transfer of either of these symbionts would be essential to generate multiple infections. Recent experimental studies have shown that *Hamiltonella* undergoes horizontal transmission during sexual reproduction (13) or by parasitoid vectors (14). Therefore, the high prevalence of coinfection may reflect the high horizontal transfer rate of *Hamiltonella*.

*Rickettsiella-Hamiltonella* coinfections may be maintained in aphid populations by their fitness effects under particular environmental conditions. Strong biotic candidates for these environmental factors are predators and parasitoids. This study showed that multiple infections by *Rickettsiella* and *Hamiltonella* caused significantly increased greenish hue levels (Fig. 2 and 3; see also Fig. S2 in the supplemental material). On green plants, aphids use their cryptic green coloration to avoid predation by *Coccinella septempunctata* (29). The rich green color of coinfected aphids seems to maximize their level of background matching, which could lead to an increase in their survival rate and in the rate of coinfected aphids in natural populations. Aphids with a *Rickettsiella*-induced green body color appear to be preferentially attacked by *Aphidius ervi* (30). Because *Hamiltonella* infection confers resistance to the parasitoid (15), *Rickettsiella-Hamiltonella*-coinfected aphids may survive better than *Rickettsiella*-infected aphids in the presence of the parasitoid. In this way, *Rickettsiella-Hamiltonella* coinfections may be maintained under the balancing selection pressures acting on red and green aphids in natural *A. pisum* populations, although these effects may be countered to some extent by the detrimental effects of the coinfection on host fitness (Fig. 4).

Meanwhile, we cannot rule out the possibility that the high prevalence of this coinfection is due to other mechanisms. For example, *Rickettsiella-Hamiltonella* coinfection may strengthen the protective effects of *Rickettsiella* against entomopathogenic fungi (27) or of *Hamiltonella* against parasitoids (15). Such combination-dependent improvements in protection have been reported for multiple infections with *Hamiltonella* and *Serratia* (47) and with *Hamiltonella* and X-type symbiont for parasitoids (11, 48). Experimental studies will be needed to test these possibilities.

**Different prevalences of *Rickettsiella* infection across geographically distinct regions.** Previous studies reported that the frequencies of *Rickettsiella* infection were approximately 7.9% in Western European *A. pisum* populations (12) and approximately 10 to 47% in North American populations (28). In contrast, in this study, no *Rickettsiella* infection was detected in Japanese populations of *A. pisum*, although we extensively surveyed as many as 1,093 insects originating from 14 sites (see Table S1 in the supplemental material). These results indicate that the infection frequencies of *Rickettsiella* are remarkably different across geographically distinct regions and that *Rickettsiella* infection was scarcely found in Japanese *A. pisum* populations.

It is conceivable that *Rickettsiella* infection is maintained by

changing the aphid body color, thereby improving its ability to escape from predators, as described above. It seems meaningful that red *A. pisum* aphids, whose body color could be affected by *Rickettsiella* infection, are absent in Japan; we have inspected thousands of *A. pisum* specimens collected in Japan and have found no red aphids to date. The body color of green *A. pisum* aphids, which are present in Japan, is not affected by *Rickettsiella* infection (12). Therefore, no improvement in the ability to escape is anticipated. This may be the reason for the failure of maintenance of *Rickettsiella* infection in Japanese natural populations. Alternatively, the differences in the frequency of *Rickettsiella* infection among regions may reflect historical processes of the organism's invasion and spread throughout the world. Further detailed analyses are needed to resolve these issues.

**Proposed candidate name.** *Rickettsiella* is an entomopathogenic bacterium that has been reported in various insects of the seven insect orders, including Hemiptera, as well as in arachnids and crustaceans (23). Our studies revealed that the *Rickettsiella* symbiont of *A. pisum* is distinctly different from previously reported *Rickettsiella* species on the basis of its phylogenetic (12), microbiological, and phenotypic traits. Therefore, we propose the designation "*Candidatus* Rickettsiella viridis" for this endosymbiotic bacterial clade. The specific name means "green" (the color induced by the bacterium in its host's body) in Latin.

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