

Interspecific symbiont transfection confers a novel ecological trait to the recipient insect

Tsutomu Tsuchida^{1,2}, Ryuichi Koga²,
Shogo Matsumoto¹ and Takema Fukatsu^{2,*}

¹Molecular Entomology Laboratory, RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

²Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan

*Author for correspondence (t-fukatsu@aist.go.jp).

In Japan, pea aphids *Acyrtosiphon pisum* mainly feed on vetch and clover, and many aphid clones produce more progeny on vetch than on clover. In this context, particular genotypes of the facultative symbiont *Regiella insecticola* enhance reproduction of infected pea aphids specifically on clover, thereby broadening the suitable food plant range of the insect. A species that is sympatric to *A. pisum*, vetch aphids *Megoura crassicauda*, are commonly found on vetch but not on clover. Laboratory rearing of *M. crassicauda* strains revealed active reproduction on vetch but substantially no reproduction on clover. Experimental transfection of *Regiella* from *A. pisum* to *M. crassicauda* by haemolymph injection established stable and heritable infection in the recipients, although no *Regiella* infection has been detected in natural populations of *M. crassicauda*. Different strains of *Regiella*-transfected *M. crassicauda* grew and reproduced on vetch, but exhibited lower fitness in comparison with corresponding uninfected aphid strains. Strikingly, the *Regiella*-transfected *M. crassicauda* exhibited improved survival and some reproduction on clover. These results suggest that *Regiella* has the potential to confer an ecological trait, adaptation to clover, on novel insect hosts, and also account for why *Regiella* is able to infect *M. crassicauda* but is scarcely found in these aphid populations.

Keywords: *Acyrtosiphon pisum*; *Megoura crassicauda*; *Regiella insecticola*; plant adaptation; fitness effects

1. INTRODUCTION

Many insects are obligatorily associated with bacterial symbionts of mutualistic nature. The best-studied case is the aphid endosymbiont *Buchnera aphidicola*, which provides essential amino acids and other nutrients deficient in the plant sap diet of the host aphids (reviewed in [1]). In addition to the obligate symbiont, a number of aphids also harbour one or a few facultative bacterial symbionts. Recent studies have revealed that these facultative symbionts confer condition-dependent fitness advantages to the host aphids, such

as heat tolerance, parasite resistance, plant adaptation, etc. (reviewed in [2]).

'*Candidatus Regiella insecticola*' (hereafter simply called *Regiella*) is a gammaproteobacterial symbiont associated with diverse aphids [3–5]. In Japan, pea aphids *Acyrtosiphon pisum* mainly feed on vetch and clover, and many aphid clones produce more progeny on vetch than on clover. Notably, *Regiella* infection was detected from aphids on clover at significantly higher frequencies than on vetch [6]. Similar associations between *Regiella* infection and clover infestation have been identified in pea aphid populations worldwide [7–9]. Experimental works showed that some *Regiella* strains are capable of improving the host fitness specifically on clover but other strains are not [10–12]. Hence, at least some *Regiella* strains are involved in plant adaptation in natural populations of *A. pisum*.

In Japan, sympatric to *A. pisum*, vetch aphids *Megoura crassicauda* are commonly found on vetch but not on clover. An extensive field survey detected no *Regiella* infection in natural populations of *M. crassicauda* [13]. Notwithstanding this, experimental transfection of *Regiella* from *A. pisum* to *M. crassicauda* by haemolymph injection established stable and heritable infection for over 50 generations [13]. Biological effects of *Regiella* transfection on *M. crassicauda* are of interest but not yet investigated.

Here we report a series of experiments to address the following questions: (i) why is *Regiella* able to infect *M. crassicauda* but is not maintained in natural aphid populations? (ii) Does *Regiella* transfection transfer the ecological trait, adaptation to clover, to the recipient *M. crassicauda*?

2. MATERIAL AND METHODS

(a) Insects

Three isofemale strains of vetch aphid *M. crassicauda*, namely HGm, TSKm and KKm, were collected from vetch *Vicia sativa* at three localities in Japan: Hongo, Tokyo, in 1999; Tsukuba, Ibaraki, in 1999; and Kyoto, Kyoto, in 2004. Diagnostic PCR and 16S rRNA gene cloning and sequencing confirmed that these strains harboured the obligate endosymbiont *B. aphidicola* but no facultative symbionts. A *Regiella*-infected strain, TUt, of pea aphid *A. pisum* was used as a donor of *Regiella*, wherein *Regiella* infection caused enhanced host fecundity specifically on white clover *Trifolium repens* [12]. A *Regiella*-cured strain of the same genotype, TUt^{amp}, was also used. These aphid strains were maintained on seedlings of the broad bean *Vicia faba* at 20°C in a long-day regimen (16 L: 8 D).

(b) Symbiont transfection

Regiella-infected strains of *M. crassicauda* were established by haemolymph transfer as described [13]. In short, haemolymph from adult TUt insects was injected into CO₂-anaesthetized third-instar HGm, TSKm and KKm insects using a glass capillary under a dissection microscope. Haemolymph of the *Regiella*-cured strain TUt^{amp} was used for control treatments. From 11–13 days after injection, nymphs of the injected insects were collected and individually reared to adulthood on broad bean plants. After collecting a sufficient number of nymphs, the adult insects were subjected to diagnostic PCR for detection of *Regiella* and *Buchnera* infections as described [13]. The *Regiella*-infected and uninfected aphid lines were maintained on seedlings of broad bean, and were regularly checked for *Regiella* infection by diagnostic PCR.

(c) Plants

Broad bean plants were used two to three weeks after sowing. Vetch and clover plants were grown from field-collected seeds and used as pre-flowering plants three to four weeks after sowing. The plants were grown on medium nutrient compost at 20°C in a long-day regimen (16 L: 8 D).

Table 1. Fitness effects of *Regiella* infection on *M. crassicauda* reared on broad bean and vetch. Asterisks indicate statistically significant differences between *Regiella*-infected and uninfected insects of the same genotype ($p < 0.05$, Mann–Whitney's *U*-test).

plant	clone	<i>Regiella</i> infection	n^a	fresh body weight of 8-day-old insect (mg) ^b	pre-reproductive period (days) ^b	fecundity (number of offspring) ^b	longevity (days) ^b
broad bean	HGm	uninfected	27	3.46 ± 0.55	8.26 ± 0.53	96.87 ± 16.55	30.37 ± 5.24
		infected	23	3.39 ± 0.58	8.52 ± 0.59	89.77 ± 14.03*	27.78 ± 4.70
	KKm	uninfected	19	3.09 ± 0.61	8.42 ± 0.51	99.37 ± 18.44	31.95 ± 4.87
		infected	20	3.18 ± 0.39	8.30 ± 0.47	81.95 ± 18.61*	25.65 ± 5.24*
	TSKm	uninfected	19	3.88 ± 0.56	8.40 ± 0.94	90.47 ± 12.79	30.84 ± 5.00
		infected	21	3.59 ± 0.38	9.00 ± 1.07	91.62 ± 6.88	30.38 ± 3.02
vetch	HGm	uninfected	14	2.45 ± 0.76	8.71 ± 0.47	78.86 ± 17.41	43.50 ± 5.13
		infected	13	1.98 ± 0.65	9.31 ± 0.85*	63.46 ± 14.86*	35.46 ± 4.33*
	KKm	uninfected	9	2.02 ± 0.66	9.22 ± 0.83	75.00 ± 14.71	40.56 ± 5.53
		infected	11	1.94 ± 0.77	9.64 ± 1.36	71.00 ± 25.07	32.36 ± 5.07*
	TSKm	uninfected	16	1.79 ± 0.83	10.75 ± 2.49	79.25 ± 24.02	40.63 ± 9.57
		infected	16	1.96 ± 0.54	9.63 ± 0.96	70.19 ± 19.75	34.06 ± 6.93*

^aTotal number of insects examined.

^bMean ± s.d.

(d) Fitness measurement

Fitness measurements were conducted 31 months for HGm (equivalent to about 94 generations), 23 months for KKm (about 70 generations) and 24 months for TSKm (about 73 generations) after the infection treatments. In all the transfected aphid strains, *Regiella* infection was stably maintained during the period. Unwinged aphids were subjected to the experiments. In order to minimize winged morph production, a limited number of aphids (*ca* 10) were maintained on a seedling of broad bean in each rearing cage. Ten-day-old adult aphids were allowed to deposit nymphs for 6 h, and the newborn nymphs were defined as 0 day old and individually transferred to a new plant of broad bean or vetch. Fresh body weight, pre-reproductive period, number of offspring and longevity of the insects were monitored every day. To examine the aphid performance on clover, the nymphs produced on broad bean plants within 6 h were kept on the same plants for 3 days. In 3-day-old aphids, presumptive unwinged nymphs and presumptive winged nymphs are distinguishable by the absence and presence of wing-pads on their thorax using a dissection microscope. After removing insects with wing-pads, the remaining unwinged-to-be nymphs were transferred to rearing cages with clover plants in 10 groups of 10 individuals for each of the *Regiella*-infected aphid strains and the uninfected aphid strains. For each of the rearing cages, longevity of the insects was monitored every day, and, if larviposition occurred, the nymphs were counted and then removed. All the experiments were conducted at 20°C in a long-day regimen (16 L:8 D). The plants were replaced by fresh ones every week.

3. RESULTS AND DISCUSSION

(a) Fitness cost of *Regiella* infection in

M. crassicauda on laboratory host plant *V. faba*

On broad bean *V. faba*, all the three genotypes of *M. crassicauda*, namely HGm, KKm and TSKm, grew well and reproduced actively. Fitness parameters for the *Regiella*-infected strains were generally equivalent or inferior to those with the uninfected strains of the same genotype. Significant reductions were identified in fecundity in HGm and KKm, and longevity in KKm (table 1).

(b) Fitness cost of *Regiella* infection in

M. crassicauda on native host plant *V. sativa*

On vetch *V. sativa*, all the three genotypes of *M. crassicauda* grew well and actively reproduced, although their fitness values were generally lower than those attained on broad bean. Fitness parameters with the *Regiella*-infected strains were generally

equivalent or inferior to those with the uninfected strains of the same genotype. Significant reductions were identified in pre-reproductive period in HGm, fecundity in HGm and longevity in HGm, KKm and TSKm (table 1).

(c) *Megoura crassicauda* quickly died on non-host plant *T. repens*, but *Regiella* infection improved survival and reproduction on the plant

On clover *T. repens*, all the three genotypes of *M. crassicauda* died promptly: nearly 80 per cent of the insects died within 2 days after transfer onto clover plants. However, a small fraction of the insects managed to survive for up to 21 days for HGm, 11 days for KKm and 42 days for TSKm (figure 1). The *Regiella*-infected strains generally exhibited better survival on clover than the uninfected strains of the same genotype. Significant differences were detected in the strains HGm and TSKm (figure 1*a,c*). Strikingly, although very small in number, two of the three *Regiella*-infected strains managed to produce some offspring on clover (figure 2). For the HGm genotype, four and 20 nymphs were produced in two of 10 clover cages with *Regiella*-infected insects while no reproduction was observed with uninfected insects (figure 2*a*). For the TSKm genotype, 11, 18, 25 and 60 nymphs were produced in four of 10 clover cages with *Regiella*-infected insects, whereas 20 and 45 nymphs were produced in two of 10 clover cages with uninfected insects (figure 2*c*).

(d) *Regiella* transfection to *M. crassicauda* conferred potential ability to survive and reproduce on non-host plant

These results illuminate an intriguing biological consequence of the *Regiella* transfection to vetch aphid *M. crassicauda*. Originally, *M. crassicauda* is almost unable to survive and reproduce on clover. However, upon *Regiella* transfection, their survival and reproduction on the non-host plant are slightly but significantly improved. Considering that the *Regiella* strain used in

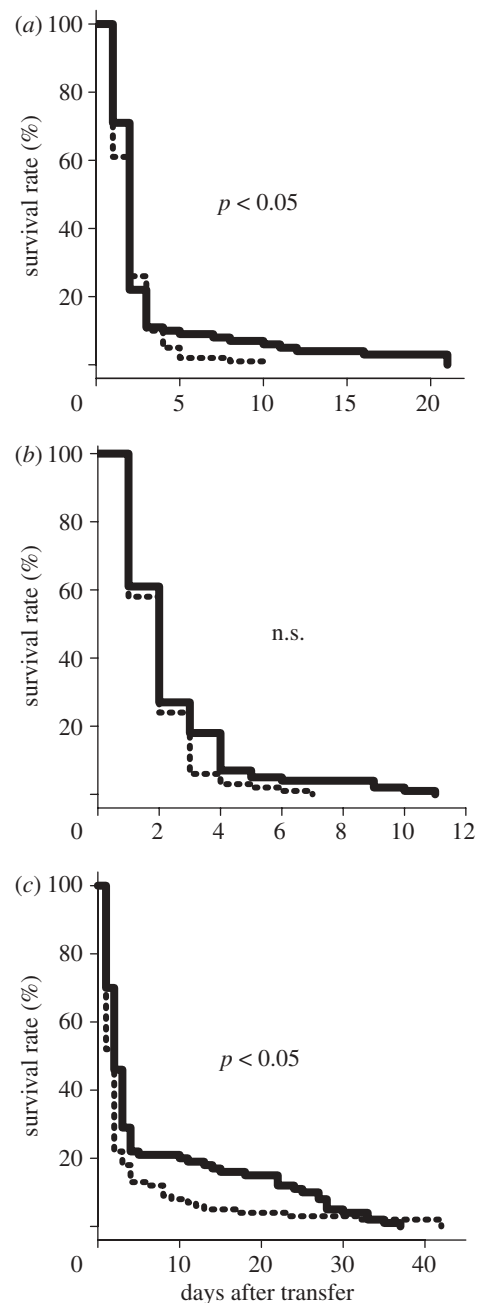


Figure 1. Survival of *M. crassicauda* on clover. (a) Strain HGm. (b) Strain KKm. (c) Strain TSKm. Survival rates of *Regiella*-infected strains and uninfected strains are shown as thick lines and dotted lines, respectively ($n = 100$ each). Statistical significance was evaluated by the generalized linear model with gamma error.

this study improves fecundity of *A. pisum* specifically on clover [12], these results can be interpreted as the transfer of an ecological trait, viability on clover, from *A. pisum* to *M. crassicauda* by symbiont transfection. Recently, a number of studies have demonstrated environment-dependent fitness advantages conferred by facultative symbionts, and the possibility has been argued that such ecological traits might be moving around between different species in association with horizontal symbiont transfers (reviewed in [2]). Our study suggests that symbiont-mediated acquisition of a novel host plant is not only theoretically but also practically feasible.

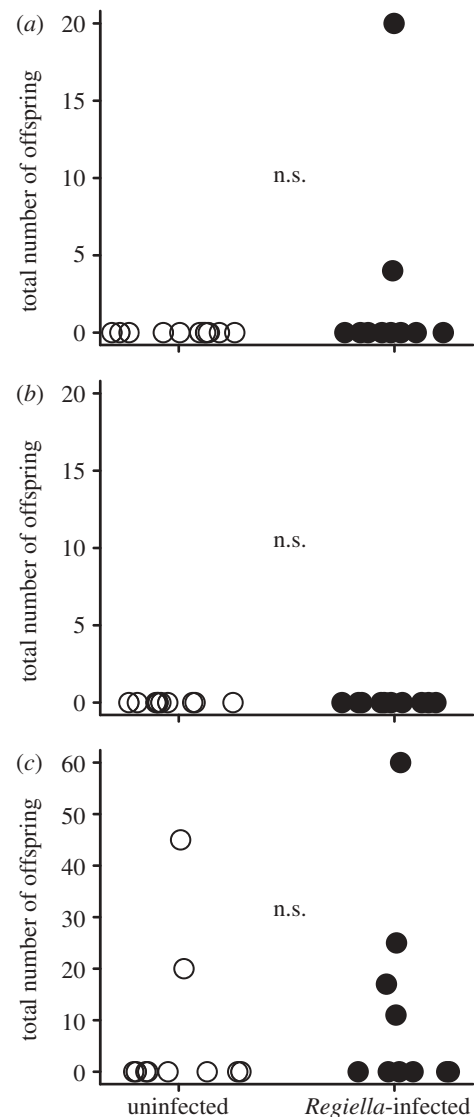


Figure 2. Reproduction of *M. crassicauda* on clover. (a) Strain HGm. (b) Strain KKm. (c) Strain TSKm. Each dot represents the total number of offspring in a rearing cage (§2). Statistical significance was evaluated by the generalized linear model with gamma error.

4. CONCLUSION AND PERSPECTIVE

The fitness data of *Regiella*-infected insects in this study, namely slightly negative effects on the native host plant *V. sativa* and only marginal positive effects on the non-host plant *T. repens*, account for the reason why *Regiella* can potentially be a symbiont of *M. crassicauda* but is scarcely found in natural populations [13]. Furthermore, it should be noted that *Regiella* infection has been detected from a diverse array of aphid species [4,5]. We expect that future experimental work on different donor–recipient combinations might identify more drastic effects of *Regiella* transfection, leading to the establishment of a tractable model system to investigate the process of symbiont-mediated acquisition of novel host plant. Previous work on *A. pisum* has demonstrated that *Regiella* is involved not only in plant adaptation but also in pathogen immunity and parasitoid resistance [14,15]. Intra- and interspecific horizontal transfers of *Regiella* infection may play some roles in the

evolutionary dynamics of these ecologically important traits in natural aphid populations.

We thank S. Koike and J. Makino for aphid rearing. This work was financially supported by Research Fellowship of Japan Society for the Promotion of Science for Young Scientists to T.T.

- 1 Douglas, A. E. 2009 The microbial dimension in insect nutritional ecology. *Func. Ecol.* **23**, 38–47. (doi:10.1111/j.1365-2435.2008.01442.x)
- 2 Oliver, K. M., Degnan, P. H., Burke, G. R. & Moran, N. A. 2010 Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu. Rev. Entomol.* **55**, 247–266. (doi:10.1146/annurev-ento-112408-085305)
- 3 Moran, N. A., Russell, J. A., Koga, R. & Fukatsu, T. 2005 Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. *Appl. Environ. Microbiol.* **71**, 3302–3310. (doi:10.1128/AEM.71.6.3302-3310.2005)
- 4 Russell, J. A., Latorre, A., Sabater-Munoz, B., Moya, A. & Moran, N. A. 2003 Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Mol. Ecol.* **12**, 1061–1075. (doi:10.1046/j.1365-294X.2003.01780.x)
- 5 Tsuchida, T., Koga, R., Meng, X.-Y., Matsumoto, T. & Fukatsu, T. 2005 Characterization of a facultative endosymbiotic bacterium of the pea aphid *Acyrtosiphon pisum*. *Microbial Ecol.* **49**, 126–133. (doi:10.1007/s00248-004-0216-2)
- 6 Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T. & Fukatsu, T. 2002 Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrtosiphon pisum*. *Mol. Ecol.* **11**, 2123–2135. (doi:10.1046/j.1365-294X.2002.01606.x)
- 7 Ferrari, J., Darby, A. C., Daniell, T. J., Godfray, H. C. J. & Douglas, A. E. 2004 Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecol. Entomol.* **29**, 60–65. (doi:10.1111/j.1365-2311.2004.00574.x)
- 8 Leonardo, T. E. & Muiro, G. T. 2003 Facultative symbionts are associated with host plant specialization in pea aphid populations. *Proc. R. Soc. Lond. B* **270**, S209–S212. (doi:10.1098/rsbl.2003.0064)
- 9 Simon, J.-C., Carre, S., Boutin, M., Prunier-Leterme, N., Sabater-Munoz, B., Latorre, A. & Bournoville, R. 2003 Host-based divergence in populations of the pea aphid: insights from nuclear markers and the prevalence of facultative symbionts. *Proc. R. Soc. Lond. B* **270**, 1703–1712. (doi:10.1098/rspb.2003.2430)
- 10 Ferrari, J., Scarborough, C. L. & Godfray, H. C. J. 2007 Genetic variation in the effect of a facultative symbiont on host-plant use by pea aphids. *Oecologia* **153**, 323–329. (doi:10.1007/s00442-007-0730-2)
- 11 Leonardo, T. E. 2004 Removal of a specialization-associated symbiont does not affect aphid fitness. *Ecol. Lett.* **7**, 461–468. (doi:10.1111/j.1461-0248.2004.00602.x)
- 12 Tsuchida, T., Koga, R. & Fukatsu, T. 2004 Host plant specialization governed by facultative symbiont. *Science* **303**, 1989–1989. (doi:10.1126/science.1094611)
- 13 Tsuchida, T., Koga, R., Sakurai, M. & Fukatsu, T. 2006 Facultative bacterial endosymbionts of three aphid species, *Aphis craccivora*, *Megoura crassicauda* and *Acyrtosiphon pisum*, sympatrically found on the same host plants. *Appl. Entomol. Zool.* **41**, 129–137. (doi:10.1303/aez.2006.129)
- 14 Scarborough, C. L., Ferrari, J. & Godfray, H. C. J. 2005 Aphid protected from pathogen by endosymbiont. *Science* **310**, 1781–1781. (doi:10.1126/science.1120180)
- 15 Vorburger, C., Gehrler, L. & Rodriguez, P. 2010 A strain of the bacterial symbiont *Regiella insecticola* protects aphids against parasitoids. *Biol. Lett.* **6**, 109–111. (doi:10.1098/rsbl.2009.0642)