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A strain of the bacterial symbiont *Regiella insecticola* protects aphids against parasitoids

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Aphids commonly harbour facultative bacterial endosymbionts and may benefit from their presence through increased resistance to parasitoids. This has been demonstrated for *Hamiltonella defensa* and *Serratia symbiotica*, while a third common endosymbiont, *Regiella insecticola*, did not provide such protection. However, this symbiont was recently detected in a highly resistant clone of the peach-potato aphid, *Myzus persicae*, from Australia. To test if resistance was indeed conferred by the endosymbiont, we eliminated it from this clone with antibiotics, and we transferred it to two other clones of the same and one clone of a different aphid species (*Aphis fabae*). Exposing these lines to the parasitoid *Aphidius colemani* showed clearly that unlike other strains of this bacterium, this specific isolate of *R. insecticola* provides strong protection against parasitic wasps, suggesting that the ability to protect their host against natural enemies may evolve readily in multiple species of endosymbiotic bacteria.

Keywords: *Aphis fabae*; endosymbiont; *Myzus persicae*; parasitoid; *Regiella insecticola*; resistance

1. INTRODUCTION

Hymenopteran parasitoids are important natural enemies of aphids and may strongly reduce their population growth (Schmidt *et al.* 2003). Despite this strong selection, there is enormous clonal variation for susceptibility to parasitoids in natural populations of aphids (Henter & Via 1995; Ferrari *et al.* 2001; von Burg *et al.* 2008; Vorburger *et al.* 2009). Some of this variation is explained by genetic differences among aphid clones (von Burg *et al.* 2008; Vorburger *et al.* 2009), but most of the variation is owing to endosymbiotic bacteria that some clones possess (Oliver *et al.* 2003). In addition to the obligate or primary endosymbiont *Buchnera aphidicola*, which serves a nutritional function (Douglas 1998), aphids may harbour a number of facultative or secondary endosymbionts. The best studied are *Hamiltonella defensa*, *Serratia symbiotica* and *Regiella insecticola* (Moran *et al.* 2005). They are faithfully transmitted from mother to offspring and have remarkable phenotypic effects on their hosts, including protection against natural enemies. *Hamiltonella defensa* and *S. symbiotica* have both been shown to increase resistance to parasitoids (Oliver *et al.* 2003), which is owing to their carrying a toxin-encoding bacteriophage (APSE) that is responsible for the defence (Oliver *et al.* 2009). *Regiella insecticola*, on the other hand, increases resistance to a fungal pathogen (Ferrari *et al.* 2004; Scarborough *et al.* 2005), but does not seem to protect against parasitoids (Oliver *et al.* 2003; Vorburger *et al.* 2009), although a comparative study by Ferrari *et al.* (2004) suggested an association between infection with *R. insecticola* and increased resistance to the parasitoid *Aphidius eadyi* in pea aphids.

In a recent study on a collection of Australian clones of the peach-potato aphid, *Myzus persicae*, von Burg *et al.* (2008) found one *R. insecticola*-infected clone to be entirely resistant to two species of parasitoids. Yet with just a single, naturally infected clone, it was not possible to infer whether the high resistance was a genetic effect or conferred by the endosymbiont. Here we report a study in which we separated these effects by experimentally infecting other aphid clones with the same isolate of *R. insecticola* and by curing the naturally infected clone with antibiotics. The results show clearly that unlike other strains of *R. insecticola*, this specific isolate strongly increases resistance to parasitoids, while also having a positive effect on aphid body size.

2. MATERIAL AND METHODS

(a) *Insects*

We worked with four pairs of aphid lines, each representing a different clone either with or without the *R. insecticola* isolate that was suspected to provide defence against parasitoids. Clone 5.15 is the resistant clone of *M. persicae* described in von Burg *et al.* (2008). It was collected in 2003 at Bacchus Marsh, Australia, and naturally harboured *R. insecticola*. Its infection with this symbiont was diagnosed by sequencing part of the 16S ribosomal RNA gene (von Burg *et al.* 2008). The sequence is deposited in GenBank (no. EF596788). We cured this clone from *R. insecticola* to create line 5.15^{R-}. For this, we injected adult females with a solution of 0.2 mg ml^{-1} of Gentamicin. Their offspring (F_1) produced on the second day after injection were reared singly until they were adult and started to reproduce. Then we sacrificed the F_1 adults and tested for the presence of *R. insecticola* by diagnostic PCR, using a primer pair specific to this endosymbiont (Tsuchida *et al.* 2006). Offspring of females that tested negative were propagated further. After all of these lines also tested negative in the F_2 and F_3 generation, we just retained one line for further use.

To transfer *R. insecticola* from clone 5.15 into three previously uninfected aphid clones, we used a microinjection protocol similar to the one described in Oliver *et al.* (2003). The recipients included two clones of *M. persicae* (5.3 and 7.9, also collected at Bacchus Marsh in 2003) and one clone of the black bean aphid, *Aphis fabae*, collected at St Margrethen, Switzerland, in 2006 (clone A06-405; Vorburger *et al.* 2009). The latter was used to test whether any protective effect of this strain of *R. insecticola* would also be expressed in other aphid species. Briefly, we anaesthetized aphids with CO_2 and punctured adults of the donor clone 5.15 to suck up the extruding haemolymph with a fine glass needle attached to a microinjection pump (FemtoJet, Eppendorf). This haemolymph was then injected into fourth instar nymphs of the receiver clones. The surviving recipients were placed individually on plants and allowed to reproduce until they died. We only retained the last few F_1 offspring they produced and tested them for infection with *R. insecticola* by diagnostic PCR after they had reproduced. Progeny of positive F_1 were propagated further and tested again in the F_2 and F_3 generations. All lines retained their acquired infection, so we reduced them again to one infected line per clone, labelled $5.3^{R5.15}$, $7.9^{R5.15}$ and A06-$405^{R5.15}$.

As parasitoid, we used *Aphidius colemani*, a species that is commonly used in the biocontrol of pest aphids and capable of parasitizing *M. persicae* as well as *A. fabae*. After a single egg is laid into an aphid nymph, the parasitoid larva develops inside the still

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Table 1. Generalized linear model results for the proportion of aphids mummified and linear model results for adult mass and daily fecundity. MS, mean squares.

		proportion mummified			adult mass			daily fecundity		
source of variation	d.f.	deviance	*F*	*p*-value	MS	*F*	*p*-value	MS	*F*	*p*-value
block	9	126.16	3.396	0.002	0.008	1.368	0.222	0.538	0.521	0.854
infection with *R. insecticola*	1	316.87	76.776	<0.001	0.038	6.435	0.014	0.002	0.002	0.969
aphid clone	3	40.08	3.237	0.028	0.020	3.298	0.026	0.297	0.287	0.835
infection × clone	3	3.03	0.244	0.865	0.005	0.865	0.464	0.630	0.610	0.611
residual	63	226.24			0.006			1.032		

active aphid. The host is only killed after completion of the larval development, when the parasitoid pupates inside its dried remains, forming a characteristic 'mummy'.

(b) *Experimental procedures*

The basic assay to measure susceptibility to parasitoids followed Henter & Via (1995): we exposed groups of aphid nymphs to parasitoids for a fixed period of time and determined the proportion of individuals that were successfully parasitized.

Before the start of the experiment, we reconfirmed the infection status of our eight lines by diagnostic PCR. We then split each line into 10 sub-lines and placed them at random positions in 10 different trays (randomized complete blocks). Sub-lines were reared at 20°C and a 16 h photoperiod on caged seedlings of either radish (*Raphanus sativum*) for *M. persicae* or broad bean (*Vicia faba*) for *A. fabae*. To avoid confounding differences among lines with environmental maternal or grand-maternal effects carried over from the stock culture, we propagated the sub-lines for two generations before testing individuals of the third sub-line generation. To start this test generation, we transferred five adult females from each sub-line to new plants to reproduce. We removed the adults again after 24 h and weighed them before disposal on a Mettler MX5 microbalance (Mettler-Toledo, Greifensee, Switzerland) to obtain an estimate of body size. Two days later, when offspring were 48–72 h old, all aphid nymphs on the plants were counted (mean colony size = 32.3 ± 4.8 s.d.). From these counts, we calculated the average number of offspring produced per adult as an estimate of daily fecundity. Then we added a single female parasitoid from our stock culture to each caged colony of aphid nymphs for 24 h. Owing to an unforeseen shortage of female wasps, we could only expose six blocks to parasitoids on the same day. The remaining four blocks were exposed on the following day, when more wasps had emerged in our stock colony. This entailed that aphid nymphs in blocks 7–10 were on average 24 h older when attacked than nymphs in blocks 1–6. Any additional variation this might have caused entered the block factor of our analyses. Ten days after exposure to parasitoids, mummies were clearly visible and counted.

(c) *Analyses*

All statistical analyses were carried out in R 2.7.1 (R Development Core Team 2008). The proportion of aphids exposed to wasps that were mummified served as our estimate of susceptibility to parasitoids and was analysed using a generalized linear model with logit link and—owing to overdispersion—quasi-binomial errors. We tested for the effects of block, infection with *R. insecticola*, clone and the infection × clone interaction. Adult body mass and daily fecundity were analysed with linear models testing for the same effects.

3. RESULTS

Infection with *R. insecticola* had a highly significant effect on aphid susceptibility to the parasitoid *A. colemani* (table 1). The originally resistant clone 5.15 became susceptible when cured from *R. insecticola*, whereas the three susceptible clones became completely or—in the case of clone A06-405—almost completely resistant when transfected with this endosymbiont (figure 1). The significant difference among the four aphid clones is largely owing to the one *A. fabae* clone being mummified at a higher rate than the three *M. persicae* clones when uninfected with *R. insecticola* (table 1, figure 1). The block effect was also significant, but there was no significant infection × clone interaction, showing that *R. insecticola* had a similar effect in different, even heterospecific genetic backgrounds.

Infection with *R. insecticola* had a positive effect on aphid adult mass, which also differed significantly among the four aphid clones (table 1, figure 2). However, these differences did not translate into variation in daily fecundity, which was similar for all clones and not affected by *R. insecticola* (table 1, figure 2).

4. DISCUSSION

We show that an isolate of the endosymbiotic bacterium *R. insecticola* from an Australian clone of *M. persicae* strongly increases aphid resistance to a parasitic wasp. Such effects have been reported previously for two other aphid symbionts, *H. defensa* and—to a lesser extent—*S. symbiotica* (Oliver *et al.* 2003). It appears that the ability to protect their host against natural enemies evolves readily in multiple species of bacterial endosymbionts. This is fascinating, if not surprising, given that under faithful vertical transmission, the evolutionary interests of host and symbiont are well aligned. Thus, *R. insecticola* should be added to the list of endosymbionts capable of defending aphids against parasitoids, even if most strains of this bacterium may not possess this ability (Oliver *et al.* 2003; Vorburger *et al.* 2009). In *H. defensa*, variation in the level of defence that different strains provide has been linked to the copy number of the toxin-encoding bacteriophage APSE, which is required for the protective phenotype (Oliver *et al.* 2009). Whether the same is true for *R. insecticola* remains to be investigated. First PCR screens did not provide any evidence for APSE in the protective strain of *R. insecticola* described here (N. Moran 2009, personal communication), but this does not exclude the possibility of other phage variants being involved.

Considering the strong benefit provided by defensive endosymbionts, it is surprising that they are not more common in aphid populations. Possibly, there are also costs of harbouring such bacteria. Here we found no evidence for this assumption, as aphids were somewhat heavier and equally fecund when infected with *R. insecticola*. However, a study by Oliver *et al.* (2008) on *H. defensa* indicates that costs may only be expressed under more realistic conditions. In the case of *R. insecticola*, we only have evidence for induced costs

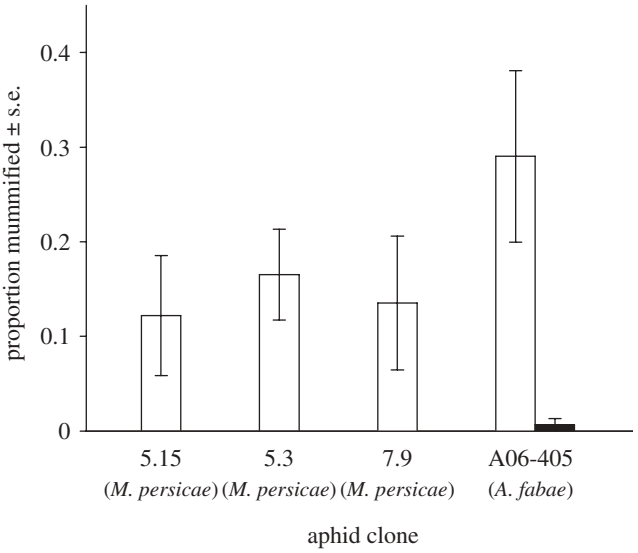

Figure 1. Susceptibility of experimental lines of aphids to the parasitoid *A. colemani*. Each bar represents the mean of 10 assays. Unfilled bars, no secondary symbiont; filled bar, *R. insecticola*.

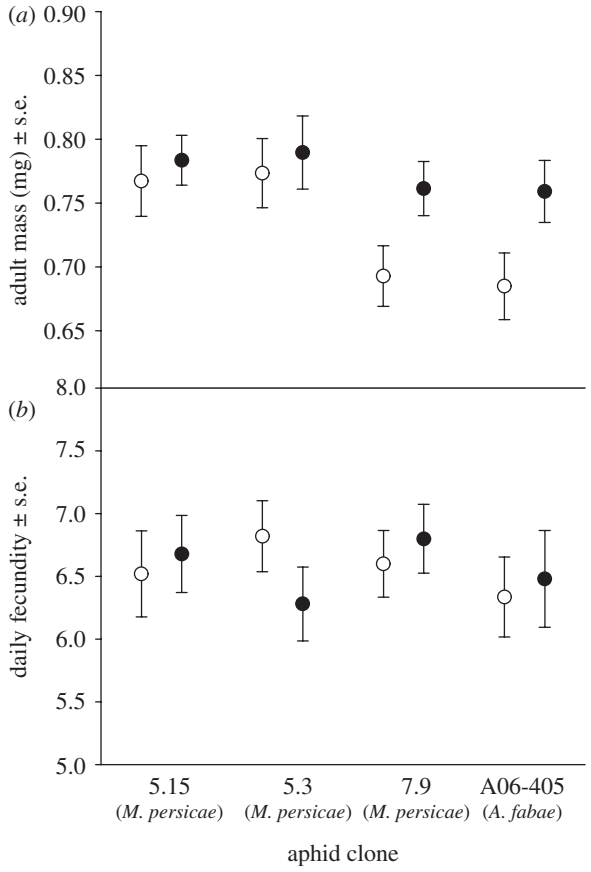

Figure 2. (*a*) Adult mass and (*b*) daily fecundity of four aphid clones in the presence and absence of the bacterial endosymbiont *R. insecticola*. Unfilled circles, no secondary symbiont; filled circles, *R. insecticola*.

of symbiont-conferred resistance, as individuals of the naturally infected clone 5.15 of *M. persicae* suffer from a strongly reduced fecundity after successfully resisting a parasitoid attack (Vorburger *et al.* 2008). This would at least reduce the benefits of harbouring *R. insecticola*.

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