PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2006) **273**, 1273–1280
doi:10.1098/rspb.2005.3436
Published online 15 February 2006

Costs and benefits of a superinfection of facultative symbionts in aphids

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Symbiotic associations between animals and inherited micro-organisms are widespread in nature. In many cases, hosts may be superinfected with multiple inherited symbionts. *Acyrthosiphon pisum* (the pea aphid) may harbour more than one facultative symbiont (called secondary symbionts) in addition to the obligate primary symbiont, *Buchnera aphidicola*. Previously we demonstrated that, in a controlled genetic background, *A. pisum* infected with either *Serratia symbiotica* or *Hamiltonella defensa* (called R- and T-type in that study) were more resistant to attack by the parasitoid *Aphidius ervi*. Here, we examined the consequences of *A. pisum* superinfected with both resistance-conferring symbionts. We found that an *A. pisum* line co-infected with both *S. symbiotica* and *H. defensa* symbionts exhibits even greater resistance to parasitism by *A. ervi* than either of the singly infected lines. Despite this added benefit to resistance, superinfections of *S. symbiotica* and *H. defensa* symbionts appeared rare in our survey of Utah *A. pisum* symbionts, which is probably attributable to severe fecundity costs. Quantitative polymerase chain reaction estimates indicate that while the density of *H. defensa* is similar in singly and superinfected hosts, *S. symbiotica* densities increased dramatically in superinfected hosts. Over-proliferation of symbionts or antagonistic interactions between symbionts may be harmful to the aphid host. Our results indicate that in addition to host–symbiont interactions, interactions among the symbionts themselves probably play a critical role in determining the distributions of symbionts in natural populations.

Keywords: endosymbiont; *Wolbachia*; multiple infection; parasitoid; proteobacteria

1. INTRODUCTION

Symbiotic associations between animals and inherited micro-organisms are widespread in nature (e.g. Buchner 1965; Douglas 1989; Werren & Windsor 2000; Terry *et al.* 2004). For example, molecular diagnostic surveys have revealed that just two bacterial symbionts, *Wolbachia* and *Cardinium*, are found in nearly 30% of certain arthropod taxa (Werren & Windsor 2000; Weeks *et al.* 2003; Zchori-Fein & Perlman 2004). It is also clear that many invertebrate hosts are superinfected, i.e. infected with multiple inherited symbionts (e.g. Buchner 1965; Unterman *et al.* 1989; Fukatsu & Nikoh 2000; Subandiyah *et al.* 2000; Dubilier *et al.* 2001; Sandström *et al.* 2001; von Dohlen *et al.* 2001; Zchori-Fein & Brown 2002; Distel *et al.* 2002; Weeks *et al.* 2003).

In general, we know little about interactions among symbionts within the superinfected hosts. Most research in this area seems to have focused on symbionts that cause cytoplasmic incompatibility (CI). In CI systems, symbionts in males depress the fitness of females of a different infection status by engineering the failure of fertilization, in this way increasing the relative fitness of females with the same infection status. In the case of symbionts causing CI, superinfections are generally predicted to invade in populations of single infections (Frank 1998). Outside of CI systems, however, the interactions of multiple symbionts in producing a host phenotype have scarcely been explored. Among the inherited symbionts that invade by providing benefits to hosts, such as nutritional supplementation (see Douglas (1998) for a review), coexistence of multiple lineages is likely to be maintained when hosts receive a net benefit that exceeds any extra costs of superinfection. Regardless of symbiont type, several factors should limit the diversity of symbionts in a single host, including competition among symbionts, increased virulence and bottlenecks experienced by symbionts during vertical transmission (Mira & Moran 2002). On the other hand, there are mechanisms that may allow for the coexistence of multiple symbionts, e.g. selective tissue tropism, and differential growth dynamics (Ijichi *et al.* 2002) or complementarity with regard to the host needs.

The pea aphid (*Acyrthosiphon pisum*) harbours several inherited secondary symbionts (SS) in addition to *Buchnera aphidicola*, the obligate primary symbiont. While *Buchnera* is necessary for successful aphid reproduction (Prosser & Douglas 1991), the role of these SS in *A. pisum* has only just begun to be explored. Already, we can attribute a diverse array of interesting phenotypes to these symbionts. For example, *Regiella insecticola* (called U-type in that study) has recently been implicated in host-plant specialization in Japanese *A. pisum* (Tsuchida *et al.* 2004), and *Serratia symbiotica* (called PASS in that study) has been implicated in thermal tolerance in North American *A. pisum* (Montllor *et al.* 2002). Further, and most relevant to the current study, isolates of *S. symbiotica* and *Hamiltonella defensa* have been shown to confer partial resistance to an important natural enemy, the parasitoid

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Received 5 November 2005
Accepted 2 December 2005

Aphidius ervi, by causing mortality to the developing wasp larvae (Oliver *et al*. 2003). The pea aphid symbiont system may be manipulated in such a way that the effect of symbiont infections may be compared in a common host genetic background. Here, we compared clonal lineages of *A. pisum* that were singly and doubly infected with *S. symbiotica* and *H. defensa*. We studied the interactions of the two symbionts in producing the parasitoid-resistance phenotype. We focused primarily on their effects on host fitness in the presence and absence of parasitoids, in an attempt to describe the costs and benefits of multiple infections under varying conditions. We also estimated the densities of primary and secondary symbionts in singly and doubly infected aphids in order to examine the interactions among symbionts. Finally, we screened over 100 field-collected *A. pisum* individuals for SS, estimating the frequencies of single and double infections. Interestingly, we note that our experimental findings can be used to explain the patterns of single and double infections in the field. Thus, we have identified a force that probably shapes the diversity of symbiotic flora harboured by single aphids.

2. MATERIAL AND METHODS

(a) Acyrthosiphon pisum *symbionts*

In addition to the obligate association with *Buchnera*, *A. pisum* (Insecta: Hemiptera: Aphididae) harbour five types of SS at intermediate frequencies: three distinct gammaproteobacterial lineages, *S. symbiotica*, *H. defensa* and *R. insecticola*, previously given the provisional labels R-type (or PASS), T-type (or PABS) and U-type (or PAUS), respectively (Unterman *et al*. 1989; Chen *et al*. 1996; Fukatsu *et al*. 2000; Darby *et al*. 2001; Sandström *et al*. 2001; Moran *et al*. 2005*b*), a *Rickettsia* (Alphaproteobacteria; Chen *et al*. 1996) and a *Spiroplasma* (Mollicutes; Fukatsu *et al*. 2001). In this study, we examine just two of these symbionts, *S. symbiotica* and *H. defensa*, which are occasionally found in the same *A. pisum* individuals in field populations. Here, we use superinfection to refer to an aphid lineage infected with two secondary symbionts: *H. defensa* and *S. symbiotica* (i.e. not to one SS plus *Buchnera*).

(b) *Study organisms*

Acyrthosiphon pisum, a polyphagous pest of herbaceous legumes (Eastop 1966), was accidentally introduced to North America from Europe around 1870 (Mackauer 1968). This aphid is cyclically parthenogenetic, allowing clonal lineages to be maintained indefinitely in the laboratory. Each clonal lineage of *A. pisum* used in this experiment consisted of descendants of a single parthenogenetic female kept in cages in a walk-in growth chamber (see Oliver *et al*. (2003) for aphid and wasp collection information). All aphid clones were maintained on *Vicia faba* (fava bean) at 20 ± 1 °C, and 16L : 8D.

Aphidius ervi (Haliday) (Hymenoptera: Braconidae) was introduced to North America in efforts to control aphid populations from Europe, and is an important natural enemy of *A. pisum* in North America (Angalet & Fuester 1977). This wasp is a solitary endoparasitoid. The adult female lays an egg inside an aphid nymph. The wasp larva then develops within the living aphid, eventually killing the host. When the host viscera have been entirely consumed, the aphid cuticle transforms into a characteristic 'mummy', usually about 8 days after oviposition. The wasp pupates within the mummy, and a free-living adult *A. ervi* emerges. The wasp culture was maintained in the laboratory at 20 ± 1 °C and 16L : 8D, on an *A. pisum* clone found to be free of secondary symbionts.

(c) *Establishment of experimental lineages*

We used a micro-injection technique, modified from Chen & Purcell (1997), to experimentally manipulate SS infection status allowing us to study the effects of particular SS in a common genetic background (see Oliver *et al*. (2003) for details). In this study, we used the singly infected *S. symbiotica* and *H. defensa* lines established in Oliver *et al*. (2003), and created a new lineage co-infected with the same isolates of *S. symbiotica* and *H. defensa*. To verify that all experimental lineages were of the same nuclear genotype, we performed a diagnostic fingerprinting technique (intersequence simple repeats or ISSR; Abbot 2001; Sandström *et al*. 2001). We also regularly screened the experimental lineages with diagnostic polymerase chain reaction (PCR) to verify SS composition (Sandström *et al*. 2001). The diagnostic PCR primers used for *H. defensa* were T1279F and 35R and the *S. symbiotica* diagnostic primers were R1279F and 35R (Russell *et al*. 2003; Russell & Moran 2006). Diagnostic PCR was conducted at 10 µl volumes using a standard reaction mix (Moran *et al*. 1999) and PCR conditions as in Sandström *et al*. (2001).

(d) *The resistance phenotype in superinfected aphids*

Previously, we reported that successful parasitism was reduced by 42% in an *A. pisum* line harbouring *H. defensa* and 23% in a line infected with *S. symbiotica* compared to an uninfected control line of the same aphid genotype (Oliver *et al*. 2003). Given the benefit conferred by each SS in singly infected hosts, we were interested in the resistance phenotype of this same *A. pisum* genotype simultaneously infected with both resistance-conferring SS. Here, we compared the rates of successful parasitism of each of the three artificially inoculated lineages (*S. symbiotica*-only, *H. defensa*-only and superinfection with both SS) of *A. pisum* against an uninfected control lineage. Thirty second instar *A. pisum* were placed on a potted *V. faba* plant in a cup cage 20–24 h prior to wasp introduction. Just prior to the experiment, wasps were given an oviposition experience by exposing them to uninfected aphids. Females with an oviposition experience were then individually assigned at random to the control or one of the experimental lineages. We removed wasps from arenas after 6 h. Arenas were incubated at 20 ± 1 °C and 16L : 8D (see Oliver *et al*. (2003) for details). We examined the arenas after 10 days and counted the numbers of mummies and surviving aphids to determine susceptibility to parasitism. Trials were conducted in two blocks. Differences between blocks were not significant and data are pooled. We analysed the proportion of successfully parasitized aphids in a logistic regression framework. All statistical analyses were performed using JMP-IN 4.0 software.

We conducted parasitism assays eight months (approximately 20 generations) after the artificial inoculation procedure to minimize any potential effects resulting from the procedure. Koga *et al*. (2003) reported detrimental effects on aphids after artificial infection with *S. symbiotica*, but these effects had attenuated by eight months post-infection.

(e) *Direct fitness benefits to resistance-conferring infections*

We previously reported that the timing of resistance in *S. symbiotica*-infected aphids occurs at 4–5 days after wasp oviposition (Oliver *et al.* 2003). We have subsequently conducted serial dissections to follow the course of developing *A. ervi* in *A. pisum* infected with *H. defensa*, and found a similar late expression of resistance (K. M. Oliver 2004 unpublished data). Given this late timing of resistance, which corresponds to a relatively large parasitoid in the aphid haemocoel, we wondered if there could be direct benefits to infection with resistance-conferring SS. To determine if parasitized aphids with resistance-conferring SS produce more offspring than parasitized uninfected controls, we performed fecundity assays of singly parasitized aphids in each of our three artificially inoculated lineages and an uninfected control. This experiment was first performed with the two singly infected lineages (*S. symbiotica* and *H. defensa*) compared with an uninfected control. A second experiment was conducted using the same protocol, adding only the superinfected lineage. In both trials, individual third instar aphids were singly parasitized by *A. ervi* in a Petri dish and then placed in groups of four aphids onto a single *V. faba* plant in a cup cage (see above) and incubated at 20 ± 1 °C and 16L : 8D. The cup cages were examined at day 6 (after parasitism) and then every 4 days. The total number of offspring and the number of surviving parasitized adults from each container were recorded at each time period. Offspring were removed at each time point to avoid confusion with later born progeny. Results for single infections and uninfected controls did not differ significantly between trials and data are pooled. In addition to depositing an egg, female *A. ervi* also injects venom that degenerates aphid ovarioles, resulting in host castration (Digilio *et al.* 2000). Our frequent observations of females producing no progeny may have been caused by host castration. The zeros in the data caused by these females resulted in non-normal fecundity distributions. We therefore use non-parametric Wilcoxon rank sum tests to analyse these data. These fecundity trials were conducted 20 months (approximately 50 generations) after the creation of the superinfected line.

(f) *Fitness costs to superinfected aphids*

Aphids infected with more than one symbiont may suffer costs associated with increased densities or due to the competitive interactions among symbionts. Casual observations in the laboratory (K. M. Oliver) suggested that *A. pisum* doubly infected with *S. symbiotica* and *H. defensa* performed poorly relative to uninfected and singly infected aphids. To address the severity of this cost, we performed fecundity assays of *unparasitized* superinfected and uninfected (no SS) *A. pisum* to quantify fecundity costs. For each replicate, four fourth instar aphids were placed on a single *V. faba* plant in a cup cage and incubated at 20 ± 1 °C and 16L : 8D. Offspring were counted every 4 days after the onset of reproduction. We also examined generation time, defined here as the time from birth to adulthood (time to first reproduction) and we measured fresh weights at adulthood of these two lines. Newborn nymphs were removed within 2 h of birth and placed on *V. faba* plants in cohorts of approximately ten aphids and maintained at 20 ± 1 °C and 16L : 8D. Eight days later, we began monitoring cages every 4 h and removed and immediately weighed reproducing aphids. These fecundity trials were conducted at 20 and 35 months after the creation of superinfected line.

(g) *Survey of* A. pisum *SS collected from alfalfa in Northern Utah*

We surveyed *A. pisum* SS collected from alfalfa near Logan, Utah (USA) in August 2003, using diagnostic PCR. Approximately 40 aphids were collected, at distances of 60 m apart, from each of three alfalfa fields. The three alfalfa fields were all within 5 km of one another. Aphids were collected and stored in 100% ethanol until DNA extraction (Engels *et al.* 1990). There were no differences among fields with regard to SS frequencies and the data shown are pooled. All the clones were screened for known *A. pisum* SS (*S. symbiotica*, *H. defensa*, *R. insecticola*, *Rickettsia* and *Spiroplasma*). Each aphid was also screened for *Buchnera* as a positive control to ensure that DNA extractions were successful. Clones testing negative for all known SS were screened with universal primers designed to amplify bacteria other than *Buchnera* (Sandström *et al.* 2001). Thirty clones, chosen haphazardly, were screened for the presence of *Wolbachia* (O'Neill *et al.* 1992), *Cardinium* (Zchori-Fein & Perlman 2004) and *Arsenophonus*, a symbiont reported from aphids and other insects (e.g. Russell *et al.* 2003). Diagnostic PCR primers were as follows: *R. insecticola*, U1279F and 35R; *Arsenophonus*, ARS1015F and 35R (Russell & Moran 2005); *Spiroplasma*, TKSSsp and 10F (Fukatsu *et al.* 2001; Russell *et al.* 2003); and *Rickettsia* (PAR4F GGCTCAGAAC GAACGCTATC and PAR1213R CACCGTCTTGCTT CCCTCTG). The reaction conditions for *R. insecticola*, *Rickettsia* and *Arsenophonus* consisted of a cycle of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min, repeated 35 times and then 72 °C for 6 min. Reaction conditions for *Spiroplasma* consisted of a cycle of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min, repeated 35 times and then 72 °C for 6 min.

(h) *Densities of* S. symbiotica *and* H. defensa *symbionts in singly versus doubly infected aphids*

To determine if SS numbers are regulated independently in superinfected aphids, we performed real-time quantitative PCR (QPCR) using a Lightcycler (Roche Molecular Biochemicals, Indianapolis IN, USA) to estimate the number of bacterial chromosomes in aphids singly and doubly infected with *S. symbiotica* and *H. defensa* SS. Aphids at age 72 ± 4 h were stored at -80 °C until DNA extraction from single individuals (Engels *et al.* 1990). We used primers designed to amplify a 244 bp fragment of the putative single copy *gyrB* gene from *S. symbiotica* (*gyrBR*-type-402R CGCTGAACAGCTACATGGAA and *gyrBR*-type-158F GCCGACCACAATTTTAGCAT) and a 201 bp fragment of *gyrB* gene from *H. defensa* (*gyrBT*-type484F TTCCT GAAATCCATCGTTCC and *gyrBT*-type685R CAAACG CAACGATCAAGAAA). Neither of these primer combinations amplifies DNA from *Buchnera*. As a negative control, we performed QPCR with the *S. symbiotica gyrB* primers on aphids infected with only *H. defensa* to ensure that primers were SS-specific (and *vice versa*). Another negative control consisting of the reaction mixture and water instead of DNA accompanied each Lightcycler run. The 20 µl QPCR reactions contained 0.5 µM of each primer, 1×SYBR Green MasterMix (Roche) and 10 pM of template DNA. T ouchdown QPCR Reaction conditions were as follows: one cycle of 95 °C for 10 min, followed by 40 cycles of 94 °C for 5 s, 68 °C for 15 s (-1 °C every three cycles down to 55 °C)

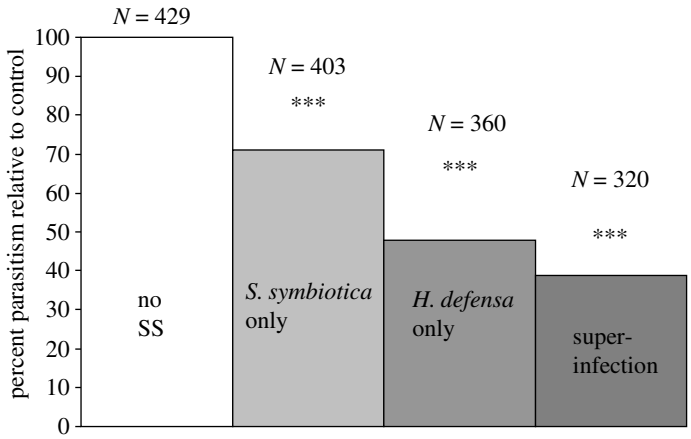

Figure 1. Back-transformed logistical regression estimates of reductions in successful parasitism of *Acyrthosiphon pisum* by *Aphidius ervi* in singly and superinfected lines relative to the uninfected control line. Numbers above columns represent the total numbers of aphids counted as alive or parasitized (***$p<0.001$).

and 72 °C for 5 s. At the end of each run, a melting curve analysis was performed, which allowed us to confirm the identity and specificity of amplified products. Separate standard curves for *S. symbiotica* and *H. defensa gyrB* were generated using two independent serial dilutions (each) of a T OPO TA plasmid vector (Invitrogen, Carlsbad, CA, USA), one containing a single fragment of the *S. symbiotica gyrB* gene and the other containing a single fragment of the *H. defensa gyrB* gene. We calculated the relative DNA concentrations of each sample with aphid EF1-alpha primers (Oliver *et al.* 2003) and calibrated SS densities accordingly. The symbiont densities were estimated approximately 20 months after creation of the superinfected line.

Koga *et al.* (2003) reported that *S. symbiotica* suppressed *Buchnera* densities. Developing wasp larvae depend upon the production of nutrients by *Buchnera* (Falabella *et al.* 2000); therefore, the degree of *Buchnera* suppression may correlate with parasitoid survival with lower *Buchnera* levels resulting in poorer parasitoid performance. To determine if *Buchnera* densities are affected by infection status, we performed QPCR to compare relative densities of *Buchnera* in aphids singly infected with *S. symbiotica* and *H. defensa* and aphids superinfected with both. We used primers designed to amplify a 176 bp fragment of the *HS70* (=dnaK) gene from *Buchnera* (*HS70F2* ATGGGTAAAATTATTGGTATTG and *HS70R2* ATAGCTTGACGTTTAGCAGG). We calculated relative DNA concentrations using aphid EF1-alpha primers to calibrate relative densities.

3. RESULTS

(a) *What is the resistance phenotype of aphids superinfected with* S. symbiotica *and* H. defensa*?*

Acyrthosiphon pisum superinfected with both *S. symbiotica* and *H. defensa* are more resistant to parasitism than those harbouring either *S. symbiotica* or *H. defensa* (figure 1). The reduction in successful parasitism ranged from 29% for the *S. symbiotica*-only line, 52% for the *H. defensa*-only line and 61% for the superinfected line. All the treatments are significantly different from one another. The regression equation comparing successful parasitism in the singly and superinfected lines with the uninfected control is $Y=-0.007+0.34\mathrm{Ssym}+0.74\mathrm{Hdef}+0.95$ Super (*S. symbiotica*-line $X^2=12.7$, $p=0.0004$, *H. defensa*-line $X^2=63.7$, $p<0.0001$, Superinfected-line $X^2=103.2$, $p=0.0004$).

We found that levels of mortality were similar (roughly 15%) between the parasitized uninfected and singly infected lines (ANOVA, $F_{2,45}=1.38$, $p=0.26$). Mortality, however, was much higher in the parasitized superinfected line (30%) compared to the uninfected and singly infected lines. Despite the increased mortality in parasitized superinfected aphids, this line produced an average of nearly three times as many surviving aphids as the uninfected control line (ANOVA, $F_{1,34}=8.6$, $p=0.006$). This indicates that superinfection results in not just fewer mummies, but in more aphids surviving attack. Fewer mummies could lead to an indirect benefit of infection if fewer parasitoids emerge to attack clone-mates, but more aphids surviving parasitoid attack raises the possibility of a direct benefit to infection.

(b) *Are there direct fitness benefits to harbouring single or multiple resistance-conferring SS isolates?*

Despite the late timing of resistance found in the *H. defensa*-only line of aphids, we found direct fitness benefits of infection—measured as cumulative fecundity—in the presence of parasitism. Third instar nymphs infected with *H. defensa* produced considerably more offspring, when parasitized compared to parasitized uninfected controls (0–18 days post-parasitism, Wilcoxon rank sum test, $p=0.0003$; table 1). Aphids with *S. symbiotica* produced similar numbers of offspring, 0–18 days post-parasitism, to the uninfected lineage (Wilcoxon rank sum test, $p=0.48$; table 1). We also found no direct fitness benefit associated with superinfected aphids despite increased survivorship of parasitized aphids. Doubly infected *A. pisum* produced comparable number of offspring relative to uninfected aphids in the presence of parasitism (Wilcoxon rank sum test, $p=0.68$; table 1).

(c) *Costs associated with double infections*

We found severe fecundity costs associated with superinfection in the absence of parasitism. At 12 days post-reproductive onset, the mean number of offspring produced by cages of uninfected aphids was 210, compared to only 38 produced by superinfected aphids (ANOVA, $F_{1,26}=120.7$, $p<0.0001$; table 2). Mortality at 12 days post-reproductive onset was much higher in superinfected

Table 1. Fecundities (0–18 days post-parasitism) of parasitized *A. pisum*. Wilcoxon rank sum tests comparing fecundities per arena (equal to four aphids) for each artificially inoculated lineage to the uninfected control lineage.

	infection status			
	uninfected	*H. defensa* only	*S. symbiotica* only	superinfected
n	34	34	34	12
mean; median fecundity	28.0; 0	87.9; 73.5	34.2; 10.0	13.7; 7.0
Wilcoxon rank sum test	N/A	$p=0.003$	$p=0.48$	$p=0.68$

Table 2. Comparison of fecundity, generation time and fresh weights of *unparasitized* superinfected versus uninfected *A. pisum*.

	infection status		
assay	uninfected	superinfected	test
cumulative fecundity (0–12 days post-reproductive onset) per cage (4 aphids per cage)	210 ($n=14$)	38 ($n=14$)	ANOVA $p<0.0001$
cumulative fecundity per aphid (0–12 days post-reproductive onset)	24.3	7.0	ANOVA $p<0.0001$
generation time (h) median, mean	211, 209 ($n=37$)	220, 214 ($n=52$)	Wilcoxon rank sum $X^2=0.002$
mean fresh weights at adulthood	3.95 g	3.15 g	ANOVA $p<0.0001$

aphids (36/64 alive = 56%) compared to uninfected aphids (61/64 alive = 95%). Cumulative fecundity per surviving aphid at 12 days was also dramatically lower in superinfected aphids compared to uninfected aphids (ANOVA, $F_{1,26}=93.8$, $p<0.0001$; table 2).

We also found that generation time was longer in superinfected aphids compared to uninfected controls (Wilcoxon rank sum $X^2=0.002$; table 2). Fresh weights at adulthood were also significantly lower in superinfected aphids compared to uninfected controls. Superinfected aphids weighed 3.15 g at adulthood compared to 3.95 g for uninfected aphids (ANOVA, $F_{1,85}=50.2$, $p<0.0001$; table 2).

(d) *Are there differences in SS density between singly and doubly infected aphids?*

The numbers of *H. defensa gyrB* gene copies per aphid were similar in singly and superinfected *A. pisum* (figure 2; ANOVA of log density per µl: $F_{1,18}=0.007$, $p=0.93$). The average number of *H. defensa gyrB* gene copies in both the *H. defensa*-only line and superinfected lines was approximately 145 000 ($n=10$). The number of *S. symbiotica* gene copies per aphid, however, was 20-fold greater in superinfected aphids compared to singly infected aphids (figure 2; ANOVA of log density per µl: $F_{1,21}=66.5$, $p<0.0001$). The number of *gyrB* gene copies in the *S. symbiotica*-only line averaged 9600 per aphid ($n=11$), but jumped to 211 000 copies in superinfected *A. pisum* ($n=12$). Qualitatively, it is interesting to note that the resistance rank (*S. symbiotica* < *H. defensa* < superinfection) corresponds broadly to total SS densities; increasing densities lead to increased resistance.

Relative *Buchnera* levels differed significantly among the two single infections and superinfection (ANOVA, $F_{2,20}=4.5$, $p=0.03$). Levels were highest in the *H. defensa*-only infected line (relative value = 1.0), intermediate in the superinfected line (relative value = 0.95) and lowest in the *S. symbiotica*-only line (relative value = 0.78). Reduced *Buchnera* levels did not correlate with reduced parasitoid survival.

(e) *Distributions of* A. pisum *SS in northern Utah*

Table 2 shows the prevalence of SS associated with 120 pea aphid clones collected from alfalfa in northern Utah. All genotypes tested positive for the aphid primary symbiont *Buchnera*. A sub-sample of 30 aphids was screened for *Wolbachia*, *Cardinium* and *Arsenophonus*; none were detected. In clones that tested negative for all described SS of *A. pisum*, we screened with universal bacterial primers that amplify bacteria other than *Buchnera*, but we did not detect any other bacteria. We found that *A. pisum* superinfected with both *S. symbiotica* and *H. defensa* were less prevalent in our survey than expected by chance (G^2 likelihood ratio test, $p=0.002$; table 3). Other superinfections occurred within expected prevalence ranges: PAR + *S. symbiotica* (G^2 likelihood ratio test, $p=0.60$) and PAR + *H. defensa* (G^2 likelihood ratio test, $p=0.21$; table 3).

4. DISCUSSION

In this study, we found that the same clonal lineage of *A. pisum* co-infected with both *S. symbiotica* and *H. defensa* exhibits even greater resistance to parasitism by *A. ervi* than clones singly infected with either (figure 1). The greater resistance phenotype may be the result of total higher densities of SS that we found in superinfected aphids (figure 2), or it could reflect the combined action of distinct defensive mechanisms of each symbiont type. The added benefit to superinfection appears surprising, however, in light of our survey of *A. pisum* SS in northern Utah, which found a lower prevalence of aphids superinfected with *S. symbiotica* and *H. defensa* SS than expected (table 3). The lower than expected prevalence of these double infections may occur if superinfections are unstable, resulting in a winnowing to single infection status. In a previous study, an *A. pisum* lineage that was

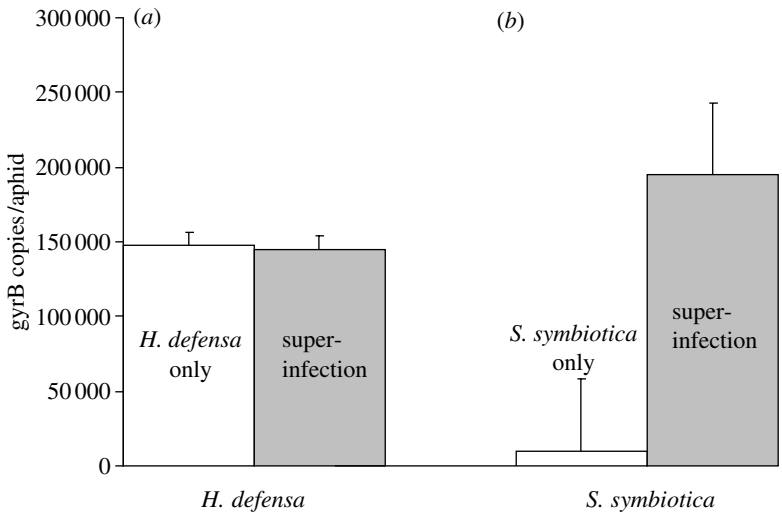

Figure 2. Comparison of SS densities in singly versus superinfected *A. pisum*. (*a*) Densities of *H. defensa* in singly infected aphids (light bar) and superinfected aphids (dark bar); (*b*) the densities of *Serratia symbiotica* in singly infected aphids (light bar) and superinfected aphids (dark bar).

naturally superinfected with *R. insecticola* and *H. defensa* was observed to undergo spontaneous loss of *R. insecticola* (Sandström *et al.* 2001). However, our superinfected *A. pisum* lineage has been maintained in the laboratory for a minimum of 80 generations.

Instead, the rarity of multiple infections with *S. symbiotica* and *H. defensa* is almost certainly a consequence of severe fitness costs, manifested as dramatically lower cumulative fecundities, longer generation times and lower weights at adulthood, associated with superinfections compared to uninfected controls (table 2). Laboratory fitness assays of single infections with either *S. symbiotica* or *H. defensa* range from slight costs to benefits (Chen *et al.* 2000; Russell & Moran 2006; K. M. Oliver, unpublished data). Koga *et al.* (2003) reported costs associated with *S. symbiotica* shortly after infection, but the detrimental effects had attenuated by eight months after infection. Our experiments were carried out in the period between eight months and 3 years after the creation of the superinfected line so costs associated with novel infection were probably minimal. Even in the presence of parasitism, there appears to be no direct fitness benefit to superinfection (table 1). This contrasts with our finding that there are clear benefits to infection in the presence of parasitism with single infections of *H. defensa* (table 2). Given the severity of the cost associated with superinfection, it may be surprising that we find them at all. One explanation is that the cost of superinfection may not be as severe in other host backgrounds. Likewise, different isolates of *S. symbiotica* and *H. defensa* may differ in degree (or kind) in regard to effects exerted on hosts, such that certain pairs of *S. symbiotica* and *H. defensa* isolates may not be as detrimental to hosts. Another possibility is that we are sampling doomed transient superinfections resulting from frequent horizontal transfer events in the field. While *A. pisum* SS are primarily transmitted vertically, phylogenetic evidence indicates that occasional horizontal transfer is necessary to explain current SS distributions in natural populations (Russell *et al.* 2003). Different SS species and isolates may rise to high frequencies due to particular benefits conferred as single infections. These benefits may reflect different ecological pressures, including parasitism by *A. ervi* as well as other natural enemies and also including abiotic factors such as heat. For example, a major benefit conferred by *S. symbiotica* infection is the improved tolerance to heat (Montllor *et al.* 2002; Russell & Moran 2006); whereas *H. defensa* isolates confer varying levels of defence against parasitoids as well as some effects on heat tolerance (Russell & Moran 2006; Oliver *et al.* 2005). Spatial and temporal heterogeneity in ecological challenges are expected to maintain polymorphism in symbiont types among aphid lineages. If alternative symbionts are maintained at high densities as single infections, the opportunity for superinfection through horizontal transfer will be maximized (although mechanisms in nature are not yet identified). This situation could result in repeated regeneration of superinfections despite major deleterious effects.

Table 3. Frequency of *A. pisum* SS collected from North Utah. $n=120$.

symbiont	frequency (%)	expected (%)	G^2 likelihood ratio test
uninfected	32.5		
S. symbiotica	18.3		
H. defensa	40		
R. insecticola	0		
Rickettsia (PAR)	5.8		
S. symbiotica+*H. defensa*	0.8	7.3	$p=0.002$
H. defensa+PAR	0.8	2.3	$p=0.21$
S. symbiotica+PAR	1.7	1.1	$p=0.60$

QPCR estimates of SS density indicate that numbers of *H. defensa* do not differ between singly and superinfected lineages (figure 2). This finding is similar to reports that *Wolbachia* densities are regulated independently in superinfected hosts (Rousset *et al.* 1999; Ikeda *et al.* 2003; Mouton *et al.* 2003, 2004). *S. symbiotica* densities, in contrast, increased 20-fold in superinfected aphids compared to aphids infected with *S. symbiotica* only. The severe fecundity cost found in superinfected aphids may

be the result of this over-proliferation of *S. symbiotica* in doubly infected hosts. Alternatively, this cost in the superinfected line could result from hostile interactions between SS or some combination of over-proliferation and antagonistic interactions. A correlation between virulence and bacterial density has been documented for *Wolbachia* infecting *Drosophila* and *Asobara* (McGraw *et al*. 2002; Mouton *et al*. 2004). There may be competing selective pressures between maintaining sufficiently high densities to ensure vertical transmission, while reducing fitness costs to hosts (Mouton *et al*. 2004).

We also used QPCR to estimate relative densities of the primary aphid symbiont, *Buchnera*, to determine if *Buchnera* suppression in SS-infected lines correlated positively with increased levels of resistance to parasitism. Although *Buchnera* densities did vary significantly, they did not do so in a pattern consistent with the hypothesis that lower *Buchnera* levels result in poorer parasitoid survival. Superinfection may still result in reduced host quality for wasps and the greatly reduced fecundity found in superinfected unparasitized aphids supports this idea.

Recently, it was reported that *H. defensa* is associated with a bacteriophage, APSE-2, which contains intact homologues of a eukaryotic toxin (CdtB or cytolethal distending toxin) that may target wasp tissue (Moran *et al*. 2005*a*). A toxin such as *CdtB*, which likely relies on bacterial invasion of host tissue for delivery, may be more effective in poorer-quality hosts, such as superinfected aphids, which may contain wasps with a compromised ability to fight off microbial attackers.

To invade a host population, vertically transmitted micro-organisms must cause their hosts to produce more infected daughters than are produced by uninfected females (Bull 1983; Werren & O'Neill 1997). In the presence of parasitism, *H. defensa* confers resistance to *A. ervi* and infected aphids produce more offspring than uninfected aphids, indicating that these symbionts can invade and persist in host populations. Despite the added resistance conferred by superinfection with *S. symbiotica* and *H. defensa*, greatly reduced fecundities would prevent invasions of the superinfection into *A. pisum* populations. Given that the interference interactions among bacterial clones are known to be common within the Gammaproteobacteria, evidence of competition between symbionts should perhaps not be surprising. Interference could result from any of the several well-characterized mechanisms, such as quorum sensing and bacteriocin production (Parret & De Mot 2002; Lerat & Moran 2004). Whatever the mechanism, these results indicate that while previous research has focused almost exclusively on the host–symbiont interaction, interactions among the symbionts themselves are likely to play a critical role in determining the distributions of symbionts in natural populations, and merit further study.

The authors are grateful to Kim Hammond, Gordon Plague, Helen Dunbar, Jacob A. Russell and Emma Huang, who provided technical assistance and consultations. The project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 2003-35302-13356 to M.S.H and N.A.M.

REFERENCES

Abbot, P. 2001 Individual and population variation in invertebrates revealed by inter-simple sequence repeats (ISSRs). *J. Insect Sci.* **1**, 1–3. (Published online 3 August 2001.)

Angalet, G. W. & Fuester, R. 1977 Aphidius parasites of pea aphid, *Acyrthosiphon pisum*, in eastern half of United-States. *Ann. Entomol. Soc. Am.* **70**, 87–96.

Buchner, P. 1965 *Endosymbiosis of animals with plant microorganisms*. New York, NY: Interscience Publishers.

Bull, J. J. 1983 *Evolution of sex determining mechanisms*. Menlo Park, CA: Benjamin/Cummings Publishing Company.

Chen, D. Q. & Purcell, A. H. 1997 Occurrence and transmission of facultative endosymbionts in aphids. *Curr. Microbiol.* **34**, 220–225. (doi:10.1007/s002849900172)

Chen, D. Q., Campbell, B. C. & Purcell, A. H. 1996 A new Rickettsia from a herbivorous insect, the pea aphid *Acyrthosiphon pisum* (Harris). *Curr. Microbiol.* **33**, 123–128. (doi:10.1007/s002849900086)

Chen, D. Q., Montllor, C. B. & Purcell, A. H. 2000 Fitness effects of two facultative endosymbiotic bacteria on the pea aphid *Acyrthosiphon pisum*, and the blue alfalfa aphid, *A. kondoi*. *Entomol. Exp. Appl.* **95**, 315–323. (doi:10.1023/A:1004083324807)

Darby, A. C., Birkle, L. M., Turner, S. L. & Douglas, A. E. 2001 An aphid-borne bacterium allied to the secondary symbionts of whitefly. *FEMS Microbiol. Ecol.* **36**, 43–50.

Digilio, M. C., Isidoro, N., Tremblay, E. & Pennacchio, F. 2000 Host castration by *Aphidius ervi* venom proteins. *J. Insect Physiol.* **46**, 1041–1050. (doi:10.1016/S0022-1910(99)00216-4)

Distel, D. L., Beaudoin, D. J. & Morrill, W. 2002 Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring bivalve *Lyrodus pedicellatus* (Bivalvia: Teredinidae). *Appl. Environ. Microbiol.* **68**, 6292–6299. (doi:10.1128/AEM.68.12.6292-6299.2002)

Dubilier, N. *et al*. 2001 Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. *Nature* **411**, 298–302. (doi:10.1038/35077067)

Douglas, A. E. 1989 Mycetocyte symbiosis in insects. *Biol. Rev.* **64**, 409–434.

Douglas, A. E. 1998 Nutritional interactions in insect–microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annu. Rev. Entomol.* **43**, 17–37. (doi:10.1146/annurev.ento.43.1.17)

Eastop, V. F. 1966 A taxonomic study of Australian Aphidoidea (Homoptera). *Aust. J. Zool.* **14**, 399–592. (doi:10.1071/ZO9660399)

Engels, W. R., Johnsonschlitz, D. M., Eggleston, W. B. & Sved, J. 1990 High-frequency P-element loss in *Drosophila* is homolog dependent. *Cell* **62**, 515–525. (doi:10.1016/0092-8674(90)90016-8)

Falabella, P., Tremblay, E. & Pennacchio, F. 2000 Host regulation by the aphid parasitoid *Aphidius ervi*: the role of teratocytes. *Entomol. Exp. Appl.* **97**, 1–9. (doi:10.1023/A:1004097427267)

Frank, S. A. 1998 Dynamics of cytoplasmic incompatibility with multiple *Wolbachia* infections. *J. Theor. Biol.* **192**, 213–218. (doi:10.1006/jtbi.1998.0652)

Fukatsu, T. & Nikoh, N. 2000 Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera). *Appl. Environ. Microbiol.* **66**, 643–650. (doi:10.1128/AEM.66.2.643-650.2000)

Fukatsu, T., Nikoh, N., Kawai, R. & Koga, R. 2000 The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* **66**, 2748–2758. (doi:10.1128/AEM.66.7.2748-2758.2000)

Fukatsu, T., Tsuchida, T., Nikoh, N. & Koga, R. 2001 Spiroplasma symbiont of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* **67**, 1284–1291. (doi:10.1128/AEM.67.3.1284-1291.2001)

Ijichi, N., Kondo, N., Matsumoto, R., Shimada, M., Ishikawa, H. & Fukatsu, T. 2002 Internal spatiotemporal population dynamics of infection with three *Wolbachia* strains in the adzuki bean beetle *Callosobruchus chinensis* (Coleoptera: Bruchidae). *Appl. Environ. Microbiol.* **68**, 4074–4080. (doi:10.1128/AEM.68.8.4074-4080.2002)

Ikeda, T., Ishikawa, H. & Sasaki, T. 2003 Regulation of *Wolbachia* density in the Mediterranean flour moth *Ephestia kuehniella*, and the almond moth, *Cadra cautella*. *Zool. Sci.* **20**, 153–157. (doi:10.2108/zsj.20.153)

Koga, R., Tsuchida, T. & Fukatsu, T. 2003 Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. *Proc. R. Soc. B* **270**, 2543–2550. (doi:10.1098/rspb.2003.2537)

Lerat, E. & Moran, N. A. 2004 Evolutionary history of quorum-sensing systems in bacteria. *Mol. Biol. Evol.* **21**, 903–913. (doi:10.1093/molbev/msh097)

Mackauer M. 1968 '*Acyrthosiphon pisum* (Harris) pea aphid (Homoptera: Aphididae)'. *Biological Control programmes against insects and weeds in Canada 1959–1968*. Technical communication, no. 4, pp. 3–11. Trinidad: Commonwealth Institute of Biological Control.

McGraw, E. A., Merritt, D. J., Droller, J. N. & O'Neill, S. L. 2002 *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proc. Natl Acad. Sci. USA* **99**, 2918–2923. (doi:10.1073/pnas.052466499)

Mira, A. & Moran, N. A. 2002 Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. *Microb. Ecol.* **44**, 137–143. (doi:10.1007/s00248-002-0012-9)

Montllor, C. B., Maxmen, A. & Purcell, A. H. 2002 Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. *Ecol. Entomol.* **27**, 189–195. (doi:10.1046/j.1365-2311.2002.00393.x)

Moran, N. A., Kaplan, M. E., Gelsey, M. J., Murphy, T. G. & Scholes, E. A. 1999 Phylogenetics and evolution of the aphid genus *Uroleucon* based on mitochondrial and nuclear DNA sequences. *Syst. Entomol.* **24**, 85–93. (doi:10.1046/j.1365-3113.1999.00076.x)

Moran, N. A., Degnan, P. A., Santos, S. R., Dunbar, H. E. & Ochman, H. 2005*a* The players in a mutualistic symbiosis: insects, bacteria, viruses and virulence genes. *Proc. Natl Acad. Sci. USA* **102**, 16919–16926.

Moran, N. A., Russell, J. A., Koga, R. & Fukatsu, T. 2005*b* 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)

Mouton, L., Henri, H., Bouletreau, M. & Vavre, F. 2003 Strain-specific regulation of intracellular *Wolbachia* density in multiply infected insects. *Mol. Ecol.* **12**, 3459–3465. (doi:10.1046/j.1365-294X.2003.02015.x)

Mouton, L., Dedeine, F., Henri, H., Bouletreau, M., Profizi, N. & Vavre, F. 2004 Virulence, multiple infections and regulation of symbiotic population in the *Wolbachia–Asobara tabida* symbiosis. *Genetics* **168**, 181–189. (doi:10.1534/genetics.104.026716)

Oliver, K. M., Russell, J. A., Moran, N. A. & Hunter, M. S. 2003 Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc. Natl Acad. Sci. USA* **100**, 1803–1807. (doi:10.1073/pnas.0335320100)

Oliver, K. M., Moran, N. A. & Hunter, M. S. 2005 Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proc. Natl Acad. Sci. USA* **102**, 12795–12800.

O'Neill, S. L., Giordano, R., Colbert, A. M. E., Karr, T. L. & Robertson, H. M. 1992 16S rRNA Phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl Acad. Sci. USA* **89**, 2699–2702.

Parret, A. H. A. & De Mot, R. 2002 Bacteria killing their own kind: novel bacteriocins of *Pseudomonas* and other gamma-proteobacteria. *Trends Microbiol.* **10**, 107–112. (doi:10.1016/S0966-842X(02)02307-7)

Prosser, W. A. & Douglas, A. E. 1991 The aposymbiotic aphid—an analysis of chlortetracycline-treated pea aphid *Acyrthosiphon pisum*. *J. Insect Physiol.* **37**, 713–719. (doi:10.1016/0022-1910(91)90104-8)

Rousset, F., Braig, H. G. & O'Neill, S. L. 1999 A stable triple *Wolbachia* infection in *Drosophila* with nearly additive incompatibility effects. *Heredity* **82**, 620–627. (doi:10.1046/j.1365-2540.1999.00501.x)

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)

Russell, J. A. & Moran, N. A. 2005 Horizontal transfer of bacterial symbionts: heritability and fitness effects in a novel aphid host. *Appl. Environ. Microbiol.* **71**, 7987–7994.

Russell, J. A. & Moran, N. A. 2006 Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proc. R. Soc. B* **273**, 603–610. (doi:10.1098/rspb.2005.3348)

Sandström, J. P., Russell, J. A., White, J. P. & Moran, N. A. 2001 Independent origins and horizontal transfer of bacterial symbionts of aphids. *Mol. Ecol.* **10**, 217–228. (doi:10.1046/j.1365-294X.2001.01189.x)

Subandiyah, S., Nikoh, N., Tsuyumu, S., Somowiyarjo, S. & Fukatsu, T. 2000 Complex endosymbiotic microbiota of the citrus psyllid *Diaphorina citri* (Homoptera: Psylloidea). *Zool. Sci.* **17**, 983–989. (doi:10.2108/zsj.17.983)

Terry, R. S. *et al.* 2004 Widespread vertical transmission and associated host sex-ratio distortion within the eukaryotic phylum Microspora. *Proc. R. Soc. B* **271**, 1783–1789. (doi:10.1098/rspb.2004.2793)

Tsuchida, T., Koga, R. & Fukatsu, T. 2004 Host plant specialization governed by facultative symbiont. *Science* **303**, 1989–1989. (doi:10.1126/science.1094611)

Unterman, B. M., Baumann, P. & McLean, D. L. 1989 Pea aphid symbiont relationships established by analysis of 16S rRNAs. *J. Bacteriol.* **171**, 2970–2974.

von Dohlen, C. D., Kohler, S., Alsop, S. T. & McManus, W. R. 2001 Mealybug β-proteobacterial endosymbionts contain γ-proteobacterial symbionts. *Nature* **412**, 433–436. (doi:10.1038/35086563)

Weeks, A. R., Velten, R. & Stouthamer, R. 2003 Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proc. R. Soc. B* **270**, 1857–1865. (doi:10.1098/rspb.2003.2425)

Werren, J. H. & O'Neil, S. L. 1997 The evolution of heritable symbionts. In *Influential passengers: inherited micro-organisms and arthropod reproduction* (ed. S. L. O'Neill, A. A. Hoffman & J. H. Werren), pp. 2–10. Oxford, UK: Oxford University Press.

Werren, J. H. & Windsor, D. M. 2000 *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proc. R. Soc. B* **267**, 1277–1285. (doi:10.1098/rspb.2000.1139)

Zchori-Fein, E. & Brown, J. K. 2002 Diversity of prokaryotes associated with *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). *Ann. Entomol. Soc. Am.* **95**, 711–718.

Zchori-Fein, E. & Perlman, S. J. 2004 Distribution of the bacterial symbiont *Cardinium* in arthropods. *Mol. Ecol.* **13**, 2009–2016. (doi:10.1111/j.1365-294X.2004.02203.x)