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Bacteriophage acquisition restores protective mutualism

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Abstract

Insects are frequently infected with inherited facultative symbionts known to provide a range of conditionally beneficial services, including host protection. Pea aphids (*Acyrthosiphon pisum*) often harbour the bacterium *Hamiltonella defensa,* which together with its associated bacteriophage *A. pisum* secondary endosymbiont (APSE) confer protection against an important natural enemy, the parasitic wasp *Aphidius ervi*. Previous studies showed that spontaneous loss of phage APSE resulted in the complete loss of the protective phenotype. Here, we demonstrate that APSEs can be experimentally transferred into phage-free (i.e. non-protecting) *Hamiltonella* strains. Unexpectedly, trials using injections of phage particles alone failed, with successful transfer occurring only when APSE and *Hamiltonella* were simultaneously injected. After transfer, stable establishment of APSE fully restored anti-parasitoid defenses. Thus, phages associated with heritable bacterial symbionts can move horizontally among symbiont strains facilitating the rapid transfer of ecologically important traits although natural barriers may preclude regular exchange.

INTRODUCTION

Temperate bacteriophages are well-known agents of horizontal gene transfer often contributing fitness-enhancing traits to bacterial hosts through phage transduction or lysogenic convergence [1]. When bacteriophages occur in microbial symbionts those benefits may extend to the animal host [2]. Terrestrial arthropods are commonly infected with heritable (i.e. maternally transmitted) facultative symbionts (HFS) that provide conditional benefits or manipulate insect reproduction in ways that favour symbiont spread [3, 4]. While relatively little is known about phage roles in HFS, some carry active phages that substantially impact the biology of both bacterial and insect hosts [5, 6]. For example, the WO and APSE phages associated with *Wolbachia* and *Hamiltonella defensa,* respectively, not only impact the within-insect abundances of their bacterial hosts, but also contribute to phenotypes expressed at the level of the insect host [7–9].

In the herbivorous insect, *Acyrthosiphon pisum* (pea aphid), infection with the bacterial symbiont *H. defensa* confers protection against the parasitic wasp *Aphidius ervi*, but only if the bacterial strain is also infected with an APSE [8]. Levels of protection against parasitism are highly variable and defense levels correlate with *H. defensa* strain and APSE variant (named APSE1, 2, etc.) with each sharing similar structural and regulatory genes, but varying in virulence cassette regions [10–12]. In aphids infected with APSE3 *H. defensa*, phage loss leads not only to the complete elimination of protection, but also increased *Hamiltonella* titres, which correlate with reduced aphid fecundity and prolonged development [9]. As phage loss disables the mutualism, phage acquisition via lateral transfer may restore functionality and thereby revive the mutualism. Phylogenetic studies indicate a history of horizontal movement among symbiont strains and species [13, 14]. Recently, infectious APSE3 particles were transferred to phage-free *Hamiltonella* cultivated *in vitro*, converting a non-protective strain into one able to inhibit the development of wasp embryos reared in culture [15]. In aphids, however, there are likely significant barriers preventing the establishment and subsequent stable vertical transmission of APSE following lateral transfer. For example, APSEs may not reach *H. defensa* populations already residing in embryos and therefore may not be inherited in the next generation of aphids. Our goal in this study was to experimentally demonstrate that APSEs can move laterally into phage-free non-protective *H. defensa* and subsequently determine if they stably re-establish in the aphid/*H. defensa* interaction and restore lost protection. Studying this process *in vivo* is

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Abbreviations: APSE, *A. pisum* secondary endosymbiont; HFS, heritable facultative symbionts; LRE, logistic regression; LRT, likelihood ratio test; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; TE, transposable element.
One supplementary figure and two supplementary tables are available with the online version of this article.

critical to understanding the contribution of horizontal phage movement to the function and evolution of this model defensive mutualism.

METHODS

Experimental transfer of APSE *in vivo*

Aphids are excellent models for studying phenotypic effects of symbiont infection due to the ability to hold aphid genotypes constant while manipulating symbiont infections. Here we extend those features to hold aphid genotype and *Hamiltonella* strain constant, while experimentally transferring APSE to a phage-free line. The four clonal *A. pisum* lines (Table S1, available in the online version of this article) used in this study were maintained on *Vicia faba* (Broad Windsor) in a Percival incubator at 20 °C on a 16 h light: 8 h dark cycle. Using microinjection, we attempted to transfer APSEs to recipient lines in three ways: the transfer of APSE3 phage particles derived from *in vitro* AS3 *Hamiltonella* cultures, APSE particles from processed aphid haemolymph (), and the transfer of unprocessed haemolymph (i.e. contains both *H. defensa* and APSE3). Microinjections were performed with groups of 20 or 30 second/third instar recipient aphid nymphs each injected with ≤1 µl of fluid via a pulled glass microcapillary needle. Groups of recipient aphids were then placed on individual *V. faba* plants held in small cages to recover for 3–4 days before being isolated and individually reared in Petri dishes with *V. faba* leaves. Offspring were screened approximately 10–15 days later with diagnostic PCR using primers specific for APSE [11]. We also confirmed that phage particles were present in all three phage transfer treatments by filtering each product through 0.2 µm syringe filters, which prevents passage of intact bacterial cells, treating the filtrate with DNase (Omega Bio-Tek) to degrade phage DNA outside of particles, then conducting DNA extractions and diagnostic PCR for APSE as above.

Effect of APSE acquisition on *Hamiltonella*-mediated protection against parasitoids

We maintained a large, laboratory culture of the braconid parasitoid, *A. ervi* (Hymenoptera: Aphidiinae) on a mixed set of susceptible pea aphid clones lacking HFS. We then conducted parasitism assays as in [8] where 20 second/third instar aphid nymphs were singly parasitized per replicate and incubated for 10 days on a single fava plant in cup cages held in a Percival incubator at 20 °C on a 16 h light: 8 h dark cycle. We then counted the number of mummies, surviving aphids and cases where aphid and parasitoid died (dual mortality).

RESULTS AND DISCUSSION

Phage APSE can horizontally transfer *in vivo* and establish in phage-free *Hamiltonella*-infected aphids with subsequent vertical transmission

In total, more than 750 individual microinjection transfers of APSE were performed. Transfer attempts were first conducted using phage particles from *in vitro* cultures or from processed aphid hemolymph (ca. 380), but mortality of injected aphids prior to reproduction was very high (74 %) and none resulted in successful establishment (Table S1). We next attempted the transfer of unprocessed haemolymph (containing both *Hamiltonella* and APSE3) from four APSE3-infected donor lines (ca. 376). Mortality prior to reproduction was lower (47 %) and we isolated 198 surviving aphids and screened their later-born offspring for APSE. Of these, 38 (19 %) first-generation offspring tested positive for APSE, but only three, all from donor clone R10, established as stable infections and were subsequently maintained for approximately 125 generations. From each of these successful transfers we established experimental lines from a single parthenogenetic female named Xfer1, Xfer2 and Xfer3. Quantitative real-time PCR was performed to examine APSE genomic copy (p2) per *Hamiltonella* (*dnaK*) cell in second/third instar nymphs using primers and reaction conditions from [11]. These ratios were similar in the three successful transfer lines ($\bar{x}$=7.4 : 1) compared to the donor line (6.7:1) (ANOVA $F_{3,22}$=0.04, P=0.99).

Given that successful APSE transfers were accomplished using unprocessed haemolymph, APSE3 detection in recipient lines could have resulted from two processes: the transfer of only APSE3 or the transfer of the APSE3 and the R10 strain of *Hamiltonella*. Transgenerational bottlenecking of *Hamiltonella* and competitive exclusion likely present strong barriers to the maintenance of >1 *Hamiltonella* strain [16]. This is supported by the rare occurrence of *Hamiltonella* coinfections in studies that used methods capable of detecting them [17]. However, to distinguish between these alternative outcomes and to rule out accidental contamination by other aphid lines, we confirmed *Hamiltonella* strain, aphid background and APSE type in our transfer lines (Xfer1-3). Donor strain R10 and recipient strain A2C are closely related and while they carry identical *recJ*, *accD*, *hrpA* and *murE* alleles, which are common loci used for sequence typing, the *ptsI* allele carries a single diagnostic SNP. We used PCR and Sanger sequencing to examine the relevant fragment of *ptsI* for strain determination (primers and reactions conditions [13]). From the three Xfer lines we only amplified the A2C allele, indicating it is likely that only APSE3 successfully established in *Hamiltonella* strain A2C. However, it remained possible that low abundance of strain R10 persisted. Genome analyses found that closely related strains of *Hamiltonella* can vary in the localization of specific transposable elements (TEs) [18]. We found that donor strain R10 contains two copies of an IS630-family TE in the 5′ end of cytochrome oxidase subunit I (Fig. S1) not present in recipient strain A2C. We then created strain-specific PCR primers (see Fig. S1 for primers and reaction conditions) that span this TE insertion to determine if both strains were present. PCR and Sanger sequencing indicated that only strain A2C is present in the Xfer lines, confirming that only APSE established after transfer (Fig. 1). Subsequent microsatellite genotyping of aphid lines [19] confirmed that Xfer lines were identical to the original A2C recipient line. Hence, these three new lines shared the same aphid genetic background and *Hamiltonella* strain of the original recipient line (A2C), but

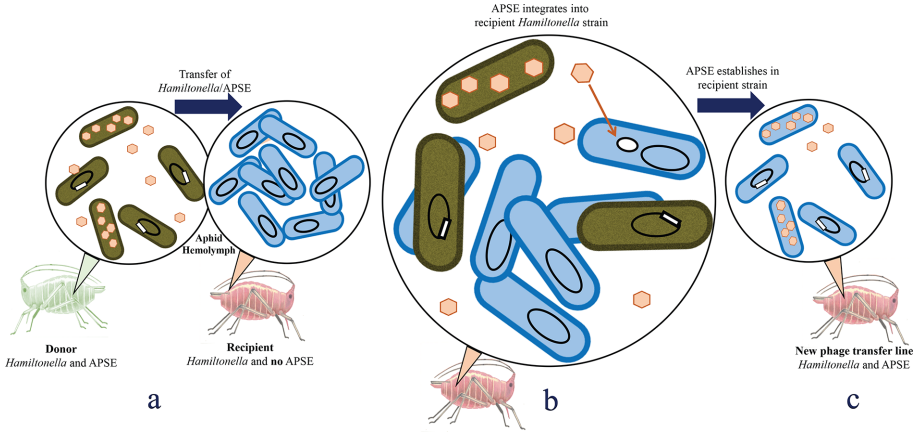

Fig. 1. (a) Microinjection was used to transfer haemolymph containing both *Hamiltonella* and APSE from donor aphids into a recipient aphid with a *Hamiltonella* strain lacking APSE. (b) Upon transfer the donor bacterial strain persists transiently and APSE infects the previously phage-free strain. Infectious APSE particles could have derived from those in the transferred aphid haemolymph or post-injection via integrated prophage from the donor *Hamiltonella* strain. The fact that particles taken from *in vitro* cultures and processed haemolymph were not stably established suggests that temporary persistence of donor strain may increase the odds of transfer. (c) Generational bottlenecking of *Hamiltonella* and possibly competition rapidly led to the persistence of only the 'recipient' *Hamiltonella* strain with a newly established APSE infection containing similar ratios of phage: bacteria compared to donor strain. Illustrations by Rebecca K. Neher.

differed only with respect to the presence of APSE infection, which was acquired from donor line R10.

In this study, APSE transfer and establishment was rare, which contrasts with observations *in vitro*, where anecdotally infectious APSE3 particles readily infect phage-free *H. defensa* [15]. While little is known about *H. defensa* transmission from mothers to embryos during parthenogenetic reproduction, they likely enter early embryonic stages simultaneously with *Buchnera* [20]. We suspect that *H. defensa* populations established in embryos prior to phage transfer may be more difficult to reach preventing establishment. Given the failure to successfully transfer only APSE particles, it is tempting to speculate that the presence of intact bacteria (which contain both integrated and encapsidated APSE) may increase the likelihood of transfer. However, we caution that with so few successful transfers it is not possible to draw a strong conclusion here. It is also unclear why the transfer of only phage particles from *in vitro* cultures and processed haemolymph resulted in increased aphid mortality relative to unprocessed haemolymph. The former contained TC100 insect cell medium (Sigma), and the latter PBS, but *H. defensa* in PBS have been successfully transferred many times without large increases in aphid mortality.

Horizontal acquisition of phage APSE rapidly restores the protective mutualism

To date, experimental work showing APSE contributions to the protective phenotype and regulation of *Hamiltonella* abundance in aphids were derived exclusively from phage-loss studies [8, 9]. Here we performed the complementary experiment, which shows that APSEs can be horizontally transferred *in vivo*.

To assess whether transfer restored protection against parasitoids, we conducted parasitism assays. The results showed that the phage-free *Hamiltonella*-infected control line A2C was susceptible to parasitism by the parasitoid *A. ervi* (Fig. 2) with 65 % of parasitized aphids (140 total) producing a parasitoid mummy; a good proxy for parasitism success [21]. In contrast, the transfer of APSE3 into the same line resulted in no mummies from 320 parasitized aphids that were generated from all three experimental lines and combined for analysis (see Table S2). The APSE3-infected donor line R10 (same APSE, but different *Hamiltonella* strain and aphid genotype) also produced no mummies, which further supported the hypothesis that the APSE variant is more important for a protective phenotype than bacterial strain.

We found unusually high rates of dual mortality (59 %), which have been reported in other studies [22]; rates are normally 20–40 %. However, dual mortality did not vary among treatments [logistic regression (LRE) Y=-0.34^{xfer}+0.12^{rec} −0.05^{don}; likelihood ratio test (LRT) P=0.68] and we used logistic regression to examine differences among treatments in the number of mummies relative to the total surviving wasps and aphids [i.e. mummies/(mummies+surviving aphids)].

Conclusion

Our results experimentally demonstrate that at least some APSE variants can be horizontally transferred between *Hamiltonella* strains infecting aphids. While phylogenetic and genomic evidence have indicated a history of

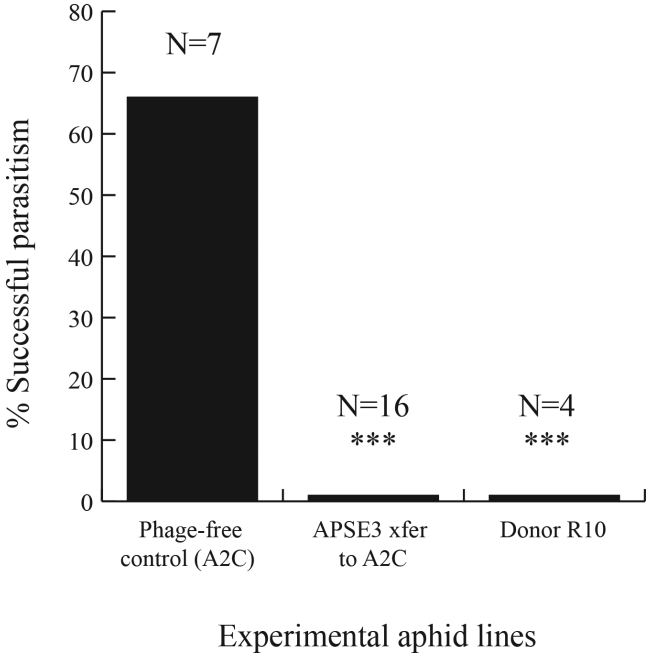

Fig. 2. APSE3 phage acquisition and establishment fully restores protective phenotype. The phage-free control line was susceptible to parasitism, while lines receiving APS3 were completely protected (0 % mummies) as was the donor line R10, which served as a positive control. Logistic regression equation *Y*=0.77A2C−7.84R10−7.7Xfer; LRT FR10=67, *P*<0.0001, LRT FR10=118, ****P*<0.0001. *N*=number of replicates.

horizontal transfer for phages APSE and WO associated with heritable bacterial symbionts (e.g. [13, 14, 23, 24]) to our knowledge this is the first experimental demonstration of lateral transfer *in vivo*. Routes of APSE transfer among *Hamiltonella* in nature are unknown, but likely mechanisms include transmission through food plants and the contaminated ovipositors of parasitoids [25, 26]. We have also found *Hamiltonella* co-infections in field-collected aphid clones, and while uncommon and likely transient, nonetheless provide ecological opportunities for APSE transfer. However, significant barriers also likely limit APSE transfer. First, phage-free *H. defensa* are uncommon in field populations due to costs associated with harbouring these strains [9] and transfer to replace an existing APSE would presumably be less common. The latter is supported by existing phylogenetic studies showing related *Hamiltonella* tend to have similar APSEs [13, 18]. Second, as noted above, natural barriers likely reduce stable vertical transmission even after successful horizontal transfer of APSE. Therefore, demonstrating APSE transfer *in vivo* confirms that phages associated with this bacterial symbiont can move ecologically important traits among hosts and contribute to the dynamism of this defensive mutualism over evolutionary timescales. However, on ecological timescales host-level selection, vertical transmission and other factors are likely more important in mutualism performance and maintenance.

Data accessibility

The supporting dataset is uploaded as part of the Supplementary Material (Table S2).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This article does not present research with ethical considerations.

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