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## Bacteriophages Encode Factors Required for Protection in a Symbiotic Mutualism

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### Abstract

Bacteriophages are known to carry key virulence factors for pathogenic bacteria, but their roles in symbiotic bacteria are less well understood. The heritable symbiont *Hamiltonella defensa* protects the aphid *Acyrtosiphon pisum* from attack by the parasitoid *Aphidius ervi* by killing developing wasp larvae. In a controlled genetic background, we show that a toxin-encoding bacteriophage is required to produce the protective phenotype. Phage loss occurs repeatedly in laboratory-held *H. defensa*-infected aphid clonal lines, resulting in increased susceptibility to parasitism in each instance. Our results show that these mobile genetic elements can endow a bacterial symbiont with benefits that extend to the animal host. Thus, phages vector ecologically important traits, such as defense against parasitoids, within and among symbiont and animal host lineages.

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Invertebrate animals are frequently infected with heritable symbionts, which can spread and persist within host populations by providing benefits to the host organism. For example, terrestrial arthropods that feed exclusively on vertebrate blood or plant sap require nutrients supplied by microbial associates to complete dietary needs (1). Recent work has also revealed that inherited symbionts can benefit diverse invertebrate hosts by providing protection against a range of natural enemies, including pathogens, predators, and parasitoid wasps (2).

In the aphid *Acyrtosiphon pisum*, infection with the inherited gammaproteobacterial symbiont *Hamiltonella defensa* confers protection against an important natural enemy, the parasitoid wasp *Aphidius ervi* (3). Female wasps deposit an egg into the aphid hemocoel, which develops within the living aphid before pupating and eventually killing the aphid host (Fig. 1A). Aphids infected with *H. defensa*, however, survive and reproduce when wasps are killed before completing development (4). Bioassays have revealed that levels of protection

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Supporting Online Material

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Materials and Methods

SOM Text

Fig. S1

Table S1

References

conferred by different strains of *H. defensa* can vary substantially (5). In the absence of parasitism, *H. defensa* is not required for aphid survival and reproduction, yet surveys of aphid populations in North America and Europe have found that significant proportions of pea aphids (40 to 70%) may be infected and that a wide variety of other aphid species also carry this symbiont (4, 6–8), which appears to confer protection in aphids other than *A. pisum* (9). In experimental populations, the proportion of *H. defensa*-infected aphids increased rapidly under pressure from parasitoid wasps, indicating that defensive benefits probably contribute to the spread of *H. defensa* in natural populations (10). The proportion of infected aphids decreased in the absence of parasitism, which suggests costs to infection that limit the spread of *H. defensa* (10).

In bacteria, including some symbionts and intracellular pathogens, lateral exchange of genetic material is typically mediated by mobile genetic elements, including transposons, plasmids, and bacteriophages (11). Such elements can introduce functions that may benefit multiple partners in a symbiotic interaction when their interests are aligned (12). *H. defensa* are frequently infected with a lysogenic lambdaoid bacteriophage called *A. pisum* secondary endosymbiont (APSE) (12–14). Several lines of evidence link APSE to the protective phenotype. First, variants of APSE have been identified among strains of *H. defensa*, and each encodes homologs of toxins that are known (or suspected) to target eukaryotic tissue (12, 13). For example, phage variant APSE-2 contains a homolog of cytolethal distending toxin (*cdtB*) (12), and aphids infected with APSE-2-carrying *H. defensa* are moderately protected from parasitism (~40% *A. ervi* mortality) (3, 5). APSE-3 encodes a tyrosine–aspartic acid repeat (YD-repeat)-containing protein, which also appears to be a toxin (13). Strains of *H. defensa* carrying APSE-3 were each found to confer high levels of protection (>85% *A. ervi* mortality) (5). The hypothesis that APSE and associated toxins are involved in aphid protection was bolstered by the results of a multi-locus study that found that the highly protective strains of *H. defensa* that carry APSE-3 are identical at 10 bacterial chromosomal markers to a third strain that lacks APSE and confers very little protection (5, 13).

The *H. defensa* chromosome contains virulence factors, including RTX (repeats in toxin) toxins, that could contribute additional protective mechanisms (12, 15). Therefore, despite analyses that showed a correlation between level of protection and presence of APSE, previous evidence could not exclude the hypothesis that variation on the *H. defensa* chromosome is the basis for the observed variation in protection to aphids. To separate effects arising from the phage, bacterial, and aphid genomes, we established *A. pisum* lines consisting of the same aphid genotype, infected with the same strain of *H. defensa*, with or without APSE (16). This possibility was presented when polymerase chain reaction (PCR) screening of the APSE P45 and *H. defensa* 16S ribosomal RNA genes revealed that some individual aphids of an *A. pisum* clone (called A1A → 5A, where A1A refers to the strain of *H. defensa*, and 5A indicates the aphid clonal background) had spontaneously lost the phage (APSE-3), retaining only *H. defensa*. We established sublines of clone A1A → 5A from single parthenogenetic females, and individuals in initial generations from each subline consistently tested either positive (now called A1A<sup>+</sup> → 5A) or negative for APSE (now A1A<sup>-</sup> → 5A).

We verified the absence of APSE in our  $A1A^- \rightarrow 5A$  line by conducting several tests. First, PCR designed to detect phage genes, including conserved genes and the YD-repeat toxin gene, did not yield amplicons in the  $A1A^- \rightarrow 5A$  strain. Second, we could not amplify fragments spanning the junction of the integrated phage genome and the integration site (13), which indicated that the phage is not integrated in line  $A1A^- \rightarrow 5A$ . Finally, a restriction digest of whole, isolated *H. defensa* chromosomes from  $A1A^+ \rightarrow 5A$  and  $A1A^- \rightarrow 5A$  lines was subjected to pulsed-field gel electrophoresis. A subsequent Southern hybridization was performed, in which DNA was transferred from the gel to a nylon membrane and exposed to a digoxigenin-labeled probe of a fragment of the APSE P45 gene [as in (17)]. In the  $A1A^- \rightarrow 5A$  line, in contrast to  $A1A^+ \rightarrow 5A$ , we did not detect the probe-bound ~40-kb fragment corresponding to APSE, demonstrating that the phage was indeed absent (fig. S1).

Using three experimental lines: (i)  $A1A^+ \rightarrow 5A$ , (ii)  $A1A^- \rightarrow 5A$ , and (iii) 5A (aphids lacking *H. defensa*), all of which share the same aphid genotype, we conducted parasitism bioassays to compare levels of parasitism and determine the contribution of the phage to the protective phenotype. Aphids of line  $A1A^+ \rightarrow 5A$  were highly resistant to parasitism by *A. ervi*; infection reduced successful wasp development by 90% compared with uninfected control line 5A (Fig. 1B) [analysis of variance (ANOVA),  $F_{1,37} = 241.1$ ,  $P < 0.0001$ ]. This protection, however, was completely lost in *A. pisum* line  $A1A^- \rightarrow 5A$ . Aphids of this line, infected with the same *H. defensa* strain but lacking APSE, suffered lethal parasitism that was ~10-fold higher than that of aphids of their phage-carrying counterparts (ANOVA,  $F_{1,44} = 396.5$ ,  $P < 0.0001$ ) and not significantly different from rates observed in the *H. defensa*-free control line 5A (ANOVA,  $F_{1,38} = 0.58$ ,  $P = 0.45$ ). These results demonstrate that required factors encoding the defensive properties attributed to this strain of *H. defensa* are located on the bacteriophage APSE-3 chromosome.

We used diagnostic PCR to screen a total of 13 *H. defensa*-infected, clonal lines of *A. pisum* maintained in the laboratory for the presence of APSE. Of the eight lines originally collected from Logan, Utah, USA, in 2003 (5 years before screening), we found that all lines retained infection with *H. defensa*, but none carried APSE (Table 1). Six of the eight *H. defensa* strains had previously carried APSE-3 (13) (Table 1), and past parasitism assays showed that some of these phage-bearing strains of *H. defensa* conferred high levels of protection against *A. ervi* (5). Most *H. defensa*-infected pea aphids in field populations also appear to contain APSE; we found that 17 out of 24 *H. defensa*-infected females carried APSE in the Utah population. Thus, in stark contrast to *H. defensa* infection in aphid lines, which is extremely stable under laboratory conditions, some phage infections are lost over time (Table 1). In laboratory lines collected at different localities and dates, retention of APSE is variable, possibly due in part to variation in the rate of spontaneous loss of APSE. Within an individual aphid carrying *H. defensa* and APSE, only some symbiont chromosomes carry an integrated phage (13); therefore, phage loss from some descendant aphid lines probably reflects a simple stochastic process through which only phage-free *H. defensa* infect certain progeny. Loss of the phage and its corresponding protective phenotype potentially explains why *H. defensa* frequencies do not approach fixation in nature, even though *H. defensa* is widespread in surveyed populations (6–8) and APSE is common in sampled *H. defensa* strains [e.g., (12, 17)].

To determine whether loss of the phage generally corresponds to loss of the protective phenotype, we conducted parasitism assays among a range of clones that were uninfected with *H. defensa*, infected with *H. defensa* and carrying APSE-3, or infected with *H. defensa* and lacking the phage. We found that all *H. defensa*-free lines are highly susceptible to parasitism, all APSE-3-carrying strains of *H. defensa* confer high levels of protection, and that the loss of the phage results in the loss of protection in every instance (Fig. 1C). Also included are results of historical assays; though they are not directly comparable, these results demonstrate that two of the same *H. defensa* strains (A2F and A2D) conferred high levels of protection when known to carry phages (Fig. 1C). These correlative results, in conjunction with the experimental evidence presented above, provide strong evidence that bacteriophages are generally required for the bulk of protection conferred to *A. pisum* by infection with *H. defensa*. Thus, *A. pisum* requires toxin-bearing viruses infecting their inherited bacterial symbionts for protection against a common natural enemy.

We performed reverse transcription quantitative PCR to estimate transcript levels of the YD-repeat protein associated with APSE-3 relative to a highly expressed bacterial gene *dnaK*. We found that transcript levels of this putative toxin were ~2.8-fold as high ( $n = 8$  aphids, mean =  $2.75 \pm 0.57$  SE) as those of *dnaK*. A previous study found that the toxin homolog (*cdtB*) associated with APSE-2 is also constitutively expressed at high levels (12). The facts that APSE is required for protection and that APSE toxin homologs are highly expressed strongly suggest a role for phage-associated toxins in the protective mechanism. However, further work is needed to determine if protection results solely from the direct action of phage-encoded factors or from a combination of factors located on both bacterial and phage chromosomes.

There are examples of bacteriophage-encoding virulence factors for pathogens, such as Shiga toxin (*stx*) in *Escherichia coli* (18) and cholera toxin in *Vibrio cholerae* (19). Here, we report the first instance of phage encoding the factors directly underlying a mutualistic symbiosis. Whereas obligate symbionts, such as *Buchnera aphidicola*, have greatly reduced and static genomes, with no evidence of ongoing horizontal gene transfer, facultative inherited symbionts, including *Wolbachia*, have been found to carry several mobile elements (14, 20–22). Our results show that mobile genetic elements can endow a bacterial symbiont with beneficial functions that extend to the eukaryotic host. In our system, the evolutionary interests of phages, bacterial symbionts, and aphids are all aligned against the parasitoid wasp that threatens them all. The phage is implicated in conferring protection to the aphid and thus contributes to the spread and maintenance of *H. defensa* in natural *A. pisum* populations.

In general, phages offer considerable potential as vectors of rapid functional change within and among diverse bacterial lineages associated with eukaryotic hosts (23). Ecologically important traits, such as defensive functions, encoded by phages may in turn facilitate invasion of new host lineages by bacteria. The presence of these labile elements increases the potential rate of evolutionary responses of hosts and bacterial symbionts in the evolutionary arms race between insects and their natural enemies.

## Supplementary Material

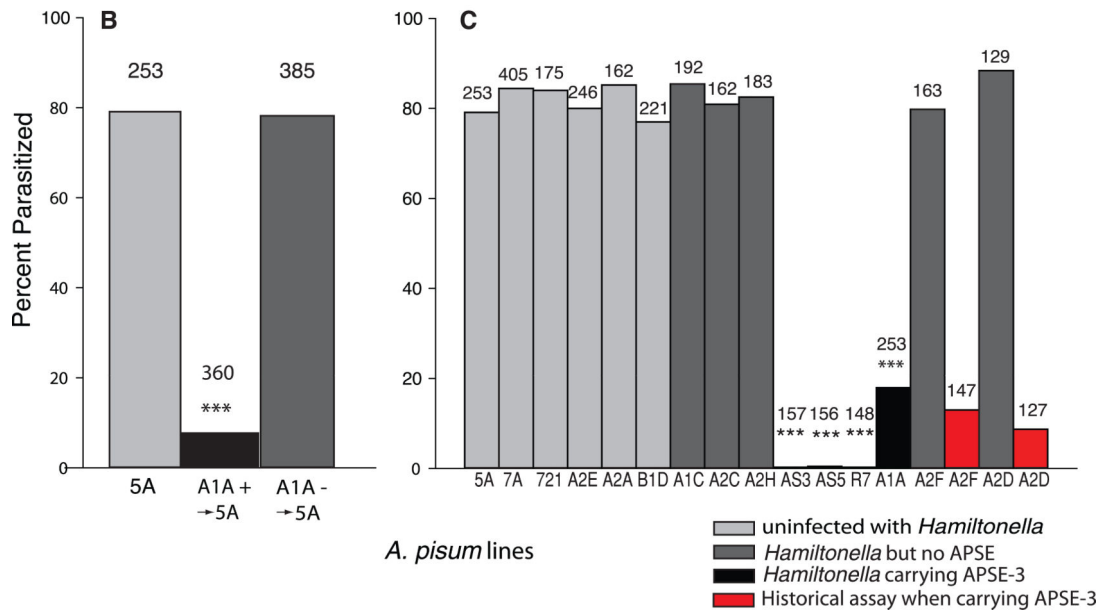
Refer to Web version on PubMed Central for supplementary material.

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**Fig. 1.** (A) Photograph of *A. ervi* parasitizing *A. pisum*. [Photo credit: Alex Wild] (B) Effect of APSE presence on the percentage of *A. pisum* successfully parasitized by the parasitoid wasp *A. ervi*. Aphid line 5A is uninfected with *H. defensa*, line A1A<sup>+</sup> → 5A is the same *A. pisum* clone infected with *H. defensa* carrying bacteriophage APSE-3, and line A1A<sup>-</sup> → 5A is the same *A. pisum* clone infected with the same strain of *H. defensa* but lacking phage APSE-3. (C) Effect of APSE on percentage of different *A. pisum* clonal lineages successfully parasitized by *A. ervi*. Numbers above columns refer to the total number of aphids counted as alive or parasitized. \*\*\**P* < 0.0001.

**Table 1**

Phage loss in *H. defensa*-infected *A. pisum* lines. Each *A. pisum* line is maintained in small cages on *Vicia faba*, either in duplicate or triplicate. Sublines were PCR screened for APSE in 2008 (16) and were found to be either all positive (+), all negative (-), or mixed (+/-). Loss of APSE-3 occurred in all six lines collected and maintained since 2003 and previously determined to carry APSE. It is unclear if lines A1C and A2C carried APSE-3 at the time of collection and subsequently lost the phage, or if they lacked APSE at the time of collection. N/A, not applicable.

<i>A. pisum</i> line	APSE in 2008	APSE in past	Phage variant and toxin	Collection year and location
82B → 5A	+	+	APSE-2 CdtB	New York, USA, 2000
A1A → 5A	+/-	+	APSE-3 YD-repeat	Utah, USA, 2003
A1A	+/-	+	APSE-3 YD-repeat	Utah, USA, 2003
A2D	-	+	APSE-3 YD-repeat	Utah, USA, 2003
A2F	-	+	APSE-3 YD-repeat	Utah, USA, 2003
A2F → 5A	-	+	APSE-3 YD-repeat	Utah, USA, 2003
A2H	-	+	APSE-3 YD-repeat	Utah, USA, 2003
A1C	-	unknown	N/A	Utah, USA, 2003
A2C	-	unknown	N/A	Utah, USA, 2003
AS3	+	+	APSE-3 YD-repeat	Utah, USA, 2007
AS5	+	+	APSE-3 YD-repeat	Utah, USA, 2007
AS5	+	+	APSE-3 YD-repeat	Utah, USA, 2007
R7	+	+	APSE-3 YD-repeat	Utah, USA, 2007