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Mobile elements create strain-level variation in the services conferred by an aphid symbiont

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Abstract

Heritable, facultative symbionts are common in arthropods, often functioning in host defence. Despite moderately reduced genomes, facultative symbionts retain evolutionary potential through mobile genetic elements (MGEs). MGEs form the primary basis of strain-level variation in genome content and architecture, and often correlate with variability in symbiont-mediated phenotypes. In pea aphids (*Acyrthosiphon pisum*), strain-level variation in the type of toxin-encoding bacteriophages (APSEs) carried by the bacterium *Hamiltonella defensa* correlates with strength of defence against parasitoids. However, co-inheritance creates difficulties for partitioning their relative contributions to aphid defence. Here we identified isolates of *H. defensa* that were nearly identical except for APSE type. When holding *H. defensa* genotype constant, protection levels corresponded to APSE virulence module type. Results further indicated that APSEs move repeatedly within some *H. defensa* clades providing a mechanism for rapid evolution in anti-parasitoid defences. Strain variation in *H. defensa* also correlates with the presence of a second symbiont *Fukatsuia symbiotica*. Predictions that nutritional interactions structured this coinfection were not supported by comparative genomics, but bacteriocin-containing plasmids unique to co-infecting strains may contribute to their common pairing. In conclusion, strain diversity, and joint capacities for horizontal transfer of MGEs and symbionts, are emergent players in the rapid evolution of arthropods.

INTRODUCTION

Heritable facultative symbionts are common and taxonomically widespread in arthropods, often playing roles in host defence against parasites and pathogens (Ballinger & Perlman, 2019; Hedges et al., 2008; Kaltenpoth & Engl, 2014; Oliver & Moran, 2009). Many defensive symbionts reside in the haemocoel, and other tissues that may be shared with additional heritable bacteria (Oliver et al., 2010; Pietri et al., 2016). The genomes of facultative defensive symbionts are intermediate in size between those of free-living bacteria and obligate symbionts, and retain diverse suites of mobile genetics elements (MGEs) that form the bulk of intraspecific (i.e., strain-level) variation (Chevignon et al., 2018; Degnan et al., 2009, 2010; Lo & Kuo, 2017; Patel et al., 2019; Scholz et al., 2020). This strain-level variation is increasingly recognized to be in important in heritable symbiosis and influences symbiont-mediated phenotypes (e.g., type or strength of protection), host–symbiont interactions (e.g., costs of housing defensive symbionts), and symbiont–symbiont

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interactions (Breusing et al., 2022; Ginete & Goodrich-Blair, 2021; Martinez et al., 2017; Oliver & Higashi, 2019; Peng et al., 2023; Smee et al., 2021). Active bacteriophages, in particular, have been shown to contribute to strain-level variation important to the function and evolution of heritable symbionts (Canchaya et al., 2003; Chafee et al., 2010; Degnan & Moran, 2008b; Kent, Funkhouser, et al., 2011; Kent, Salichos, et al., 2011; Lynn-Bell et al., 2019; Moran et al., 2005).

Aphids (Hemiptera: Aphidoidea) are a group of sap-feeding insects with a propensity toward symbiosis. Beyond their obligate, nutritional *Buchnera* symbionts, aphids variably associate with nine facultative symbionts, with demonstrated roles in thermal tolerance and defence against parasitoids and pathogens (Asplen et al., 2014; Guo et al., 2017; Heyworth et al., 2020; Higashi et al., 2023; Lukasik et al., 2013; McLean et al., 2020; Oliver et al., 2003; Russell & Moran, 2006; Scarborough et al., 2005; Schmid et al., 2012). Among these is *Hamiltonella defensa*, which defends against parasitoids and is estimated to occur in about one-third of aphid taxa (Oliver & Higashi, 2019; Vorburger, 2014; Wu et al., 2022; Zytynska & Weisser, 2016). The symbiotic habit in *H. defensa* (*Yersiniaceae*; *Gammaproteobacteria*) evolved tens of millions of years ago (Russell et al., 2003), as evidenced by its monophyly with two other aphid-associated symbionts, *Regiella insecticola* and *Fukatsuia symbiotica* (Patel et al., 2019). Its modestly sized, ~2.1 Mb genome, is comprised of numerous MGEs, making up ~20% of its genomic content (Chevignon et al., 2018; Degnan et al., 2009). Among these elements is an intact toxin-encoding bacteriophage called APSE, which is required for the symbiont's anti-parasitoid defence (Moran et al., 2005; Oliver et al., 2009). APSEs are double-stranded DNA phages with mosaic genomes comprised of four functional modules; two modules (1 and 2) encode DNA metabolism genes, one is associated with virion assembly (Module 4) and another with virulence genes (Module 3; Boyd et al., 2021; Rouïl et al., 2020; van der Wilk et al., 1999). APSE virulence modules collectively encode homologues of three eukaryotic toxins thought to play key roles in symbiont-based defences: cytolethal distending toxin, shiga-like toxins, and YD-repeat proteins (Degnan & Moran, 2008a; Oliver & Perlman, 2020).

The pea aphid, *Acyrthosiphon pisum*, is a species complex consisting of more than 10 biotypes specialized on different herbaceous legumes (Peccoud et al., 2009). Across disparate populations of the alfalfa (*Medicago sativa*) biotype, *H. defensa* is the most common facultative symbiont (Ferrari et al., 2012; Russell et al., 2013). Specific strains of *H. defensa* are also enriched in this biotype. Among sampled North American populations, most strains reside in one of five clades (named A–E) identified by multi-locus typing of specific single-copy orthologues (Degnan & Moran, 2008b; Henry et al., 2013; Peng et al., 2023; Smith et al., 2021). Annotated genomes have been reported for five isolates (i.e., those 'isolated' from a single aphid clone) that represent strains residing in three *H. defensa* clades (A-clade isolates 5AT and NY26, D-clade isolates AS3 and A2C, and E-clade isolate ZA17; Chevignon et al., 2018; Degnan et al., 2009). Separately derived isolates from the same *H. defensa* clades (e.g., A or D) show high similarity in content, including MGE composition and genome organization, while isolates derived from different clades (e.g. A vs. D) vary in MGE content and genome organization (Chevignon et al., 2018).

Strain level variation in *H. defensa* is associated with important biological features. For example, *H. defensa* strain, including APSE variant, correlates with variable protection against the pea aphid's most common parasitoid, *Aphidius ervi* (Hymenoptera: Braconidae; Angalet & Fuester, 1977; McLean & Godfray, 2015; Oliver et al., 2005; Oliver & Higashi, 2019). Isolates of A- and E-clade *H. defensa*, associated with APSE2 and APSE8, respectively, and each encoding very similar alleles of cytolethal distending toxin (*cdtB*), confer moderate anti-parasitoid protection (ca. 45% reduction in parasitoid mortality compared with uninfected controls; Chevignon et al., 2018; Degnan & Moran, 2008a, 2008b; Martinez et al., 2018; Oliver et al., 2005, 2009). D-clade *H. defensa*, on the other hand, typically house APSE3, which encodes YD-repeat (YDp, polymorphic Rhs family) toxin homologues and confer high to complete (≥85% parasitoid mortality compared with controls) protection against *A. ervi*. These correlations support the hypothesis that APSE variant is the best predictor of *H. defensa*'s protective phenotype (Oliver & Higashi, 2019). However, experimentally partitioning the contributions of phage and bacterial chromosomes to host protection remains challenging given that these elements are co-inherited, that is, related *H. defensa* isolates typically carrying the same APSE variants. Experimental efforts to isolate APSE roles have been restricted to phage loss and phage gain studies in D-clade *H. defensa*, which can spontaneously lose their APSE3s and then be experimentally re-introduced (Brandt et al., 2017; Lynn-Bell et al., 2019; Oliver et al., 2009; Weldon et al., 2013). The presence of distinct APSE variants among otherwise highly similar same-clade *H. defensa* strains would provide an elegant means to more broadly examine phage and bacterial contributions to anti-parasitoid protection.

Strain level variation is also associated with patterns of enriched co-infections of pea aphid facultative symbionts revealing a 'cryptic community structure' (Peng et al., 2023). The most strongly enriched association occurs with B-clade *H. defensa* and the most common strain of *F. symbiotica*. We originally hypothesized that these co-infections would be maintained by host-level selection on infection costs and benefits, but

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experimental studies involving these specific strains found *F. symbiotica* provided no identifiable benefits in single or co-infection contexts (Doremus et al., 2018; Doremus & Oliver, 2017). We had next hypothesized that there may be metabolic cooperation (e.g., syntropy) between these two symbionts as seen between degraded obligate symbionts and facultative symbionts (Meseguer et al., 2017). However, a recent analysis that examined metabolic complementarity among pea aphid facultative symbionts revealed little potential for between cooperation between *H. defensa* and *F. symbiotica* (Peng et al., 2023). Another possibility is that the extensive variation in MGE content among *H. defensa* strain clades, including gene content associated with extra-chromosomal plasmids, contributes to the stability of this association (Chevignon et al., 2018). For example, these elements may encode factors that mediate competitive interactions among related bacteria (Dawid et al., 2007; Speare et al., 2018; Thappeta et al., 2020).

Here we present annotated genomes for two B-clade strains, and one C-clade strain, with a focus on MGE content that underlies symbiont-mediated phenotypes. These build on prior genomics of strains from the A, D, and E clades (Chevignon et al., 2018; Degnan et al., 2009) totalling eight pea aphid *H. defensa* genomes spanning all five common strain clades. Genome comparisons with additional diagnostics of APSE and *H. defensa* revealed that otherwise highly similar B-clade strains of *H. defensa* contained different APSE phages, indicating repeated horizontal transfer not observed in other clades. Bioassays showed that variation in APSE virulence modules was the major determinant of the protective phenotype. We also identified bacteriocin-containing plasmids that may contribute to common coupling of B-clade *H. defensa* and *F. symbiotica*.

EXPERIMENTAL PROCEDURES

Information on three focal *H. defensa*

Isolates used to generate B-strain genomes were derived from two alfalfa biotype pea aphid clones, 5D and MI47, which were naturally co-infected with *H. defensa* and *F. symbiotica*. Clone 5D and associated symbionts were used in previous experimental studies, where sublines infected only with *H. defensa* (5DH) or *F. symbiotica* (5DX) were created (Doremus & Oliver, 2017) and used to generate whole genome sequences (this study; Patel et al., 2019). For clone MI47, we observed that *F. symbiotica* was not faithfully vertically transmitted. This allowed us to isolate offspring and establish a new subline infected with only a B-clade strain of *H. defensa* (MI47H), which was then used as source material for genomics. To produce a C-strain genome, we used an isolate from aphid clone MI12 that was naturally infected only with *H. defensa*. These strains were grown in vitro to generate template free of aphid and *Buchnera* DNA using previously published protocols (Boyd et al., 2021; Brandt et al., 2017; Chevignon et al., 2018; Patel et al., 2019). Select data derived from these genomes were used in recent studies on APSE genomics (Boyd et al., 2021) and metabolic interactions among facultative symbionts (Peng et al., 2023).

Genome assemblies and annotations

Culture-extracted DNA was sent to the Drexel University College of Medicine Genome Core Facility for SMRTBell™ fragment library construction using Long-Insert Genomic DNA, followed by SMRT sequencing using PacBio Sequel system. Data were collected on a SMRTCell™ with 107,939 reads (N50 read length = 27,215) for isolate 5DH; and 115,370 reads (N50 read length = 39,073) for isolate MI47H; and; and 114,000 reads (N50 read length = 38,583) for isolate MI12H (see Table S1 for additional sequencing metrics). We generated de novo assemblies using the Hierarchical Genome Assembly Process (HGAP.2) algorithm in the SMRT Portal (version 2.3.0) with default settings following (Chevignon et al., 2018; Patel et al., 2019).

The assembled genome sequences of all three *H. defensa* isolates were submitted for coding sequence (CDS) prediction to the National Center for Biotechnology Information (NCBI) Prokaryotic Genomes Annotation Pipeline (Tatusova et al., 2016) and Rapid Annotation Subsystem Technology tool kit (RASTtk; Brettin et al., 2015). All prediction software was last accessed in 2022 unless otherwise noted. Predictions from each pipeline per isolate were merged and compared using BEACON (Kalkatawi et al., 2015). SMRT sequencing produces occasional random bp deletions in homopolymeric runs (Ross et al., 2013), which we corrected manually as in Patel et al. (2019). Pseudogenes were called when <80% of a given CDS was complete compared with those of nearest relatives. We used RNAmmer (Lagesen et al., 2007) and tRNA scanner (Schattner et al., 2005) to predict rRNAs and tRNAs, respectively. BlastKOALA and IMG-ER were used to predict metabolic pathways in *H. defensa* (Kanehisa et al., 2016; Markowitz et al., 2012). Phage island predictions were made using PHASTER (Arndt et al., 2016) and transposable elements were searched for using ISsaga (Varani et al., 2011) and ISfinder (Siguier et al., 2006); last accessed 2018. Plasmid islands on the main chromosomes of each isolate were analysed manually. We also aligned genomes of the three focal *H. defensa* and APSE using progressiveMauve in Geneious version 11 (www.geneious.com) and visualized key comparisons using easyFig v2.2.2 (Sullivan et al., 2011; see Table S2 for accession numbers).

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Average nucleotide identity and average amino acid identity (AAI) among *H. defensa* isolates were determined using the Environmental Microbial Genomics calculator at http://enve-omics.ce.gatech.edu/ (Rodriguiz-R & Konstantinidis, 2016).

Screening for bacteriocin-harbouring plasmids across *H. defensa*

With the observation that our two B-strain *H. defensa* genomes uniquely harboured extrachromosomal plasmids containing bacteriocins, we attempted a broader survey for these plasmids across numerous isolates derived from all five *H. defensa* clades (A–E). Toward this end, we screened aphids with previously typed (A–E) *H. defensa* strains through 10 μL diagnostic PCR (polymerase chain reaction), using primers specific to an ABC transporter gene that only occurs on these plasmids (pHDMI47.3 and pHD5D.3 see Results section). Reactions containing 5 μL 2× Quanta SYBR green PCR master mix, 1 μL of forward primer (cpF1 5′ ACTTAAGGGAACGCTGAGCA 3′), 1 μL of reverse primer (cpR1 5′ GAACAACCCAGATTGCCACT 3′) and 1 μL of DNA template used for diagnostic PCR using an AnalytikJena qTOWER^3 thermal cycler. The cycling conditions involved an initial denaturation step at 95°C for 5 min followed by 40 cycles of 95°C (10 s), 60°C (10 s), and 72°C (10s) followed by a melting curve analysis to confirm the intended target was amplified.

Phylogenetic reconstruction of *H. defensa* and APSE

To reconstruct phylogenetic relationships of *H. defensa* derived from various aphid species, including sequenced isolates 5DH, MI47H, and MI12H from pea aphids, we used partial sequences of seven single-copy orthologous genes (Degnan & Moran, 2008b) with the closely related symbionts *F. symbiotica* and *R. insecticola* included as outgroups. Nucleotide sequences were downloaded from NCBI, or were produced via Sanger sequencing (see Table S2 for accession numbers). We also Sanger sequenced four loci (Table S2), using these data to infer a phylogeny of only pea aphid alfalfa biotype-associated *H. defensa*, with those from the whitefly *Bemisia tabaci* used as an outgroup (Table S2). We, further, estimated phylogenetic trees for APSE based on loci representing modules 1 (P51), 2 (P3), and 4 (P19) of the mosaic APSE genome and inferred a phylogeny from four loci that included only pea aphid alfalfa biotype-associated *H. defensa* using *B. tabaci* as an outgroup (Table S2). We also estimated phylogenetic trees for APSE based on loci representing modules 1 (P51), 2 (P3) and 4 (P19) of the mosaic APSE genome (Boyd et al., 2021; Degnan & Moran, 2008b). Primers and reaction conditions for all loci except P19 are found in Degnan and Moran (2008b). For P19, primer sequences were P19-12159F: 5′CGCGCAATATTGGGTTTATT 3′ and P19-12925R: 5′ GGATCAAGCGCATTGACTAA 3′ using the same reaction conditions as Degnan and Moran (2008b). Additional APSE sequences were downloaded from NCBI (see Table S2 for accession numbers).

For all phylogenies, sequences were aligned in MAFFT in Geneious v11 using the L-INS-I algorithm, prior to manual curation. After finding best-fit partition models (Table S3) using ModelFinder (Kalyaanamoorthy et al., 2017), we inferred maximum likelihood phylogenies with 10,000 ultrafast bootstrap (UFBoot2) replicates using IQ-TREE v2.0 (Hoang et al., 2018; Minh et al., 2020).

Parasitism assays to determine the protective phenotype of *H. defensa*

To isolate phage contributions to the protective phenotype, we performed parasitism assays in pea aphid clones harbouring B-clade *H. defensa* with distinct APSE variants. By controlling for *H. defensa* genotype, we tested the hypothesis that different phages or toxins will result in variable levels of parasitism (Table 1). We also examined levels of protection of an E-clade *H. defensa* (ZA17) containing an APSE with an identical *$cdtB_2$* allele, and highly similar virulence module to those of an APSE from B-clade *H. defensa* 5DH. We predicted that the presence of similar variants/toxins in *H. defensa* from different clades will produce a similar level of anti-parasitoid protection. We also characterized protection levels of C-clade *H. defensa* (MI12H), which have not previously been reported. The MI12H isolate carries an APSE9 with a CdtB toxin that differs from those found in ASPE2 and 8 (Boyd et al., 2021).

To conduct parasitism assays, we first created experimental aphid lines (Table 1) by either microinjection of *H. defensa*/APSE into an uninfected aphid clone, or selective antibiotic curing of *H. defensa* without harming *Buchnera* (Doremus & Oliver, 2017). Experimental aphid lines were reared at 20 ± 1°C on a 16-h light:8-h dark cycle on fava bean (*Vicia faba*), 'Broad Windsor' plants. Using previously published diagnostics (Russell et al., 2013), we screened for all seven pea aphid facultative symbionts to ensure only the expected symbionts were present. We maintain a large laboratory colony of *A. ervi* established from pea aphid mummies (pupating parasitoids) collected primarily from Dane County, Wisconsin (USA), and maintained on a mixture of susceptible pea aphid lines uninfected with facultative symbionts. Field-collected *A. ervi* were added to the colony once or twice per year to maintain genetic variability in wasps. Adult wasps were provided a diet of honey and water, and held under the same environmental conditions as aphids.

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TABLE 1 Information about experimental pea aphid lines used in parasitism assays.

Aphid subline name	H. defensa isolate (collection info)	Aphid genotype (collection info)	H. defensa clade	APSE variant and toxin gene	Creation method
5DH-5DAB	5DH (WI, 2012)	5D (WI, 2012)	B	APSE8 (*cdtB*$_2$)	Natural
WI49H-5DAB	WI49H (WI, 2017)		B	APSE10 (*sltxA*$_2$)	Injection
ZA17-5DAB	ZA17 (PA, 2010)		E	APSE8 (*cdtB*$_2$)	Injection
MMP1-5DAB	MMP1 (PA, 2018)		B	APSE3 (*ydp*)	Injection
5DAB	None		None	None	Antibiotics
MI47H	MI47H (MI, 2018)	MI47 (MI, 2018)	B	APSE1 (*sltxA*$_1$)	Natural
MI47HAB	None		None	None	Antibiotics
MI12H-AS3	MI12H (MI, 2018)	AS3 (UT, 2007)	C	APSE9 (*cdtB*$_3$)	Injection
AS3AB	None		None	None	Antibiotics
2184H	2184H (WI, 2018)	2184 (WI, 2018)	C	APSE9 (*cdtB*$_3$)	Natural
2184AB	None		None	None	Antibiotics

Note: All aphids were collected on alfalfa (*Medicago sativa*) and clonal lines were established from single parthenogenetic females.
Abbreviations: MI, Michigan; PA, Pennsylvania; UT, Utah; WI, Wisconsin (all locales in USA). Grey shading highlights experimental lines that share aphid genotype.

All parasitism assays were conducted at least five generations after antibiotic-mediated symbiont removal, or transfer via microinjection, to minimize potential effects of antibiotic treatment or recent symbiont acquisition (Niepoth et al., 2018). To characterize the protective phenotype of each *H. defensa* isolate, we examined rates of successful parasitism, determined by the number of mummies produced, an established proxy (Oliver et al., 2012) using previously published protocols (Martinez et al., 2018). In brief, second- or third-instar pea aphid nymphs were singly parasitized by a mated, female *A. ervi* and placed in cohorts of 20 on single, potted fava bean seedlings contained within 16 oz. ‘cup cages’ with a mesh lid, and then held in an environmental chamber at 20°C on a 16-h day:8-h night schedule. Ten days later, the numbers of aphids surviving (resistant), mummified (susceptible), and otherwise dead (mortality of both aphid and parasitoid) were determined. Likelihood ratio tests (LRT) using a logistic regression model were performed to determine statistical significance in parasitism assays. We also conducted post hoc Tukey–Kramer (honestly significant difference) HSD tests on arcsine transformed values of parasitism success (mummies/(mummies + surviving aphids) to account for multiple comparisons when needed. All statistics were conducted using JMP PRO versions 16 or 17 (SAS Institute Inc., Cary, NC, 1989–2023).

RESULTS

Summary of ‘B’ and ‘C’ strain *H. defensa* genomes.

Similar to other *H. defensa* isolates (Chevignon et al., 2018), the main chromosomes, and select extrachromosomal plasmids of 5DH, MI47H and MI12H could not be circularized due to palindromic regions at contig ends generated by the *de-novo* assembly.

The genome of isolate MI12H (NCBI CP023987.1), which is the first strain of C-clade *H. defensa* sequenced, was broadly similar in size (2.13 Mb), GC content (40.6%), core gene inventories and extrachromosomal plasmid number to *H. defensa* strains from other clades (Table 2, S1, and Figure S1). Its plasmids (pHDMI12.1 and pHDMI12.2) were similar to pHDZA17.1 and pHDZA17.2 from the E strain ZA17 (79.2% and 87.5%, respectively; Chevignon et al., 2018). APSE9 was present as a prophage in the MI12H isolate as previously reported (Boyd et al., 2021).

Isolates 5DH and MI47H (NCBI identifiers NZ_CP021663.1 and NZ_CP022932.1, respectively) represent the first whole genomes for B-clade *H. defensa*. These were highly similar in genome size (2.03 vs. 2.04 Mb), GC content (both 40.4%), total CDS count (1968 vs. 1977), and the number of pseudogenes on the main chromosome (128 vs. 139; Table S1). The two B-clade isolates shared nearly identical gene inventories with an average AAI of 99.32% (Table S4). Only a small number of intact genes in isolate MI47H were absent (Table S5a) or pseudogenized (Table S5b) in isolate 5DH. Conversely, all intact genes in 5DH were present in MI47H, with only a few being pseudogenized (Table S5c). One notable difference between these two isolates was that *metN*, part of the transporter complex for methionine transport was pseudogenized in MI47H. Additional work is needed to understand the biological significance of this difference.

Both B-strain genomes carried five homologous plasmids, which is more than is typical for *H. defensa* (Table 2). Except for plasmids pHDMI47.2 and pHD5D.2, which are similar to pHDAS3.1 from D-clade *H. defensa* (Chevignon et al., 2018), the remaining

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TABLE 2 Genome features of sequenced *H. defensa* from pea aphids.

	5AT	NY26	MI47H	5DH	MI12H	AS3	A2C	ZA17
Clade	A	A	B	B	C	D	D	E
Chromosome size (Mb)	2.11	2.12	2.03	2.02	2.13	2.05	2.00	2.10
Plasmid number	1	0	5	5	2	2	3	3
Coding sequence (CDS) on chromosome (pseudogenes)	2241 (203)	2243 (282)	1977 (139)	1968 (128)	2087 (208)	2181 (200)	2116 (156)	2185 (170)
CDS on plasmid (pseudogenes)	65 (7)	N/A	261 (57)	264 (54)	137 (20)	230 (28)	231 (22)	185 (28)
rRNA	9	9	9	9	9	9	9	9
tmRNA	1	1	1	1	1	1	1	1
tRNA	42	42	43	42	42	43	43	42
Prophage islands	22	22	19	19	28	13	13	21
Plasmid islands	11	11	7	7	9	7	7	7
APSE variant	APSE2	APSE2	APSE1	APSE8	APSE9	APSE3	none	APSE8

Note: Shaded columns indicated newly sequenced genomes.

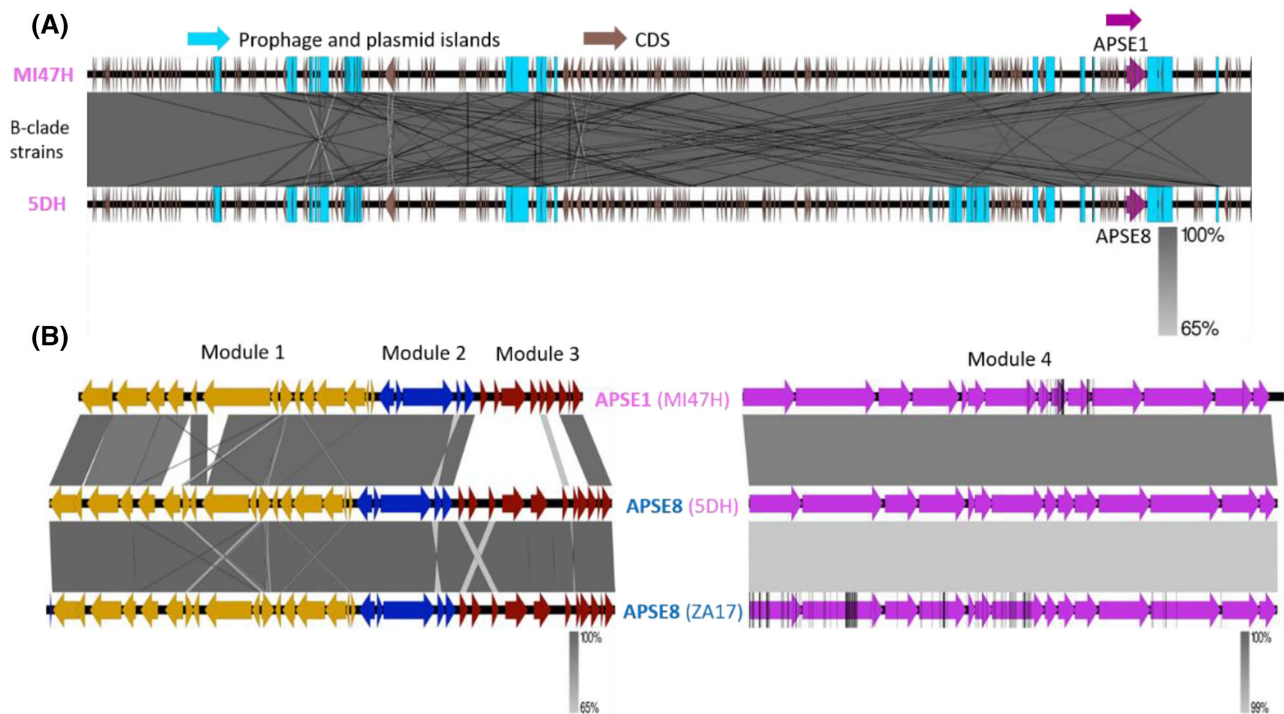

FIGURE 1 *Hamiltonella defensa* and APSE genome alignments. (A) Two B-clade *H. defensa* isolates are overall similar in gene content and order except for housing distinct APSE variants (magenta arrows). (B) The APSE8 haplotype from *H. defensa* isolate 5DH is most similar to an APSE8 haplotype from isolate ZA17 across Modules 1, 2, and 3 (virulence cassette), but is more similar to APSE1 across module 4 (magenta). The vertical lines in Module 4 show single nucleotide polymorphisms (SNPs) in APSE1 (MI47H) and APSE8 (ZA17) relative to the APSE8 from isolate 5DH. The colour of the *H. defensa* isolate name (e.g., MI47H) matches the A–E strain clade designation in Figure 2 (pink, B-clade *H. defensa* strains). Similarly, the colour of the APSE variant name (e.g., blue, APSE8) matches the variant designation in Figure 2. CDS, coding sequence.

B-strain plasmids were distinct. Plasmids pHDMI47.1 and pHD5D.1 are conjugative plasmids that encode a putative *higAB* toxin–antitoxin system and also carry genes for the transportation of glycerol and fatty acid biosynthesis. Plasmids pHDMI47.3 and pHD5D.3 encode bacteriocins and components of a type-1 secretion system (T1SS). Common in gram-negative bacteria, TISS are tripartite protein secretion systems that function in pathogenesis and mediating bacterial competition (Klein et al., 2020). These B-strain plasmids

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encode multiple copies of four distinct bacteriocins and repeats of an ATP-binding cassette (i.e. ABC transporters) and membrane fusion protein (*hlyD*; Figure S2). The third TISS component, an outer membrane protein (TolC) is found on the bacterial chromosome of most *H. defensa*, including B-clade strains. Some of the bacteriocins have homology to bacteriocin-like peptides (blp) found in the opportunistic pathogen *Streptococcus pneumoniae* that play roles in regulating bacterial competition (Dawid et al., 2007).

Whole genome alignments showed similar genome organization of the two B-strain isolates 5DH and MI47H, including that of MGEs (Figure 1A). For example, each possessed 21 prophage/prophage islands, 12 plasmid islands, and 154 TEs (Tables S6–S8). Among the latter, TE/insertion sequence class IS630 was the most prolific (Table S8). Although the location and orientation of the intact APSE bacteriophages were similar, the key difference between the two B-clade strains was that isolate 5DH carried an APSE8 prophage, whereas MI47H carried an APSE1 (Figure 1A). In contrast to within-strain similarity, B-clade MGE content and genome architecture differed markedly from that of strains occupying other *H. defensa* clades (Figure S3). For example, the number, type, and location of TEs, as well as prophage and plasmid islands differed between B and C clade strains (Tables S6–S8).

MGEs may underlie common co-infection of B-clade *H. defensa* with *F. symbiotica*

We next examined the 5DH and MI47H genomes for unique content that may underlie the strong association of B-clade *H. defensa* and *F. symbiotica*. We found that *H. defensa* strains spanning all five clades shared nearly identical gene inventories for nutrient intake, metabolism, and housekeeping function (Figure S1). This is consistent with our recent finding that complementary metabolic potential is unlikely to explain this relationship (Peng et al., 2023). Hence, it is unlikely that B-clade strains engage in less exploitation competition with *F. symbiotica* compared with other *H. defensa* strains (e.g., Kreimer et al., 2012).

We next considered MGE features unique to B-clade *H. defensa* that may contribute to the maintenance of this co-infection. While other differences are present (Tables S7 and S8), we were most intrigued by the plasmid-associated bacteriocins and TISS machinery, given their roles in mediating interspecies bacterial competition (Klein et al., 2020). While experimentally demonstrating these hypothesized roles was beyond the scope of our study, we screened for the presence of these bacteriocin-containing plasmids (e.g., pHDMI47.3 or pHD5D.3) across more than 200 isolates spanning all five *H. defensa* clades. Using DNA template from field-collected aphid samples with previously characterized *H. defensa* strain assignments, we found these plasmids were present in 98% of B-clade isolates ($N = 135$), but in no other *H. defensa* ($N = 89$; Table S9). Among aphids harbouring B-clade strains with varying co-infection contexts (i.e., no coinfection, *H. defensa* + *F. symbiotica*, or *H. defensa* + other symbionts), we found no correlation between plasmid presence and the presence/identity of symbiont co-residents. Specifically, all seven B-strain isolates living under single infection screened positive for the bacteriocin-containing plasmids. These plasmids were also prevalent (i.e., 97%; $N = 127$) among B-strain isolates co-infecting with *F. symbiotica* (Table S9). Hence, while bacteriocin-bearing plasmids are not a perfect predictor of *F. symbiotica* presence, this correlation may be a fruitful area for future study.

Relatedness among *H. defensa* strains

To understand the evolutionary history of pea aphid-*H. defensa* associations, we generated maximum likelihood phylogenies of *H. defensa* isolates, including those from B- (5DH, MI47H) and C-clade strains (MI12H). While most isolates derived from alfalfa biotype pea aphids can be assigned to one of five clades (A–E: Figure S4), isolates from particular strains are closely related to strains inhabiting other aphid species. This illustrates that *H. defensa* likely colonized pea aphids on multiple occasions. A maximum likelihood phylogeny restricted to pea aphid isolates resulted in a similar tree topology, showing relatedness between the B and D clades and, separately, the C and E clades (Figure 2A).

Repeated horizontal transfer of APSE among B-clade *H. defensa*

We next examined APSE phage distributions across a range of studied *H. defensa* isolates (Figure 2A). Based on genome sequences and additional multi-locus typing we found that A-clade isolates (e.g., 5AT and NY26) were associated with APSE2, and D-strain isolates (e.g., AS3) carried APSE3. In contrast, two APSE variants (APSE9 and 11) were found in C-strain isolates. Phage diversity; however, was greatest among B-clade *H. defensa*, which is associated with four APSEs (1, 3, 8, and 10; Figure 2A). A Fisher's exact test (FET) indicates a significant association between *H. defensa* strain clade (A–E) and number of associated APSE variants (FET, $F = 109.6$, df $= 24$, $p < 0.001$).

In some cases, entire APSEs appear to have been exchanged. For example, the APSE3 in B-strain isolate MMF12H is similar across all four modules at examined loci compared with APSE3 from D-strain isolate AS3. In other cases, we see signatures of recombination between APSE variants, as well as intra-module

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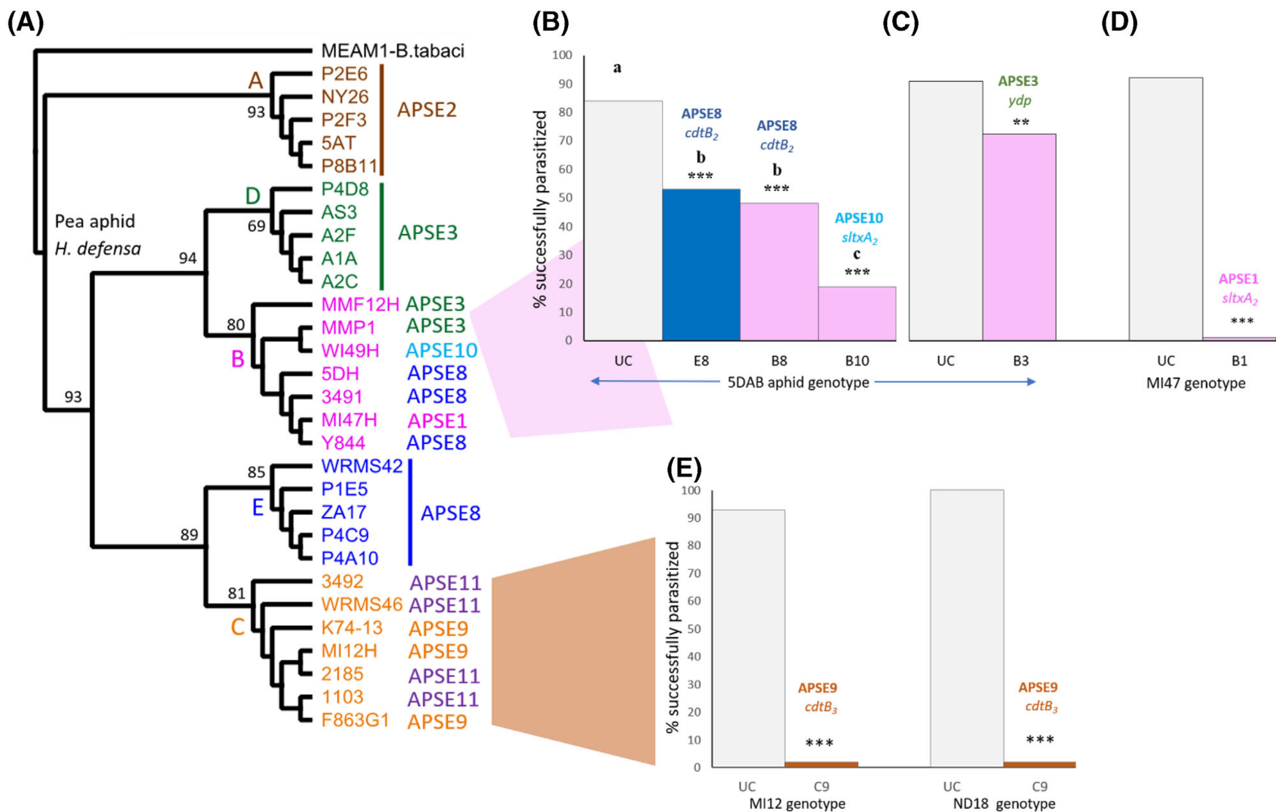

FIGURE 2 (A) Relatedness of *H. defensa* isolates inferred from maximum likelihood analysis with APSEs mapped onto tips of phylogeny. Colours are used to indicate bacterial strain and APSE variant with similar *H. defensa*/colours used when frequently co-occurring. (B–E) Results of parasitism assays in experimental aphid lines harbouring varying *H. defensa*/APSE. Percent of pea aphids successfully parasitized (i.e., mummified) by *A. ervi* in bioassays is shown on the *y*-axis. (B) Assay using one E-strain isolate and two B-clade strains with varying APSEs. (C) Assay in the same pea aphid clone ('5DAB aphid genotype') using one B-strain isolate with APSE3. (D) Assay in different aphid clone using B-strain isolate with APSE1. E) Assays in two additional aphid clones using a C-strain *H. defensa* with APSE9. In (B–E), 'UC' (Uninfected Control) refers to aphid sublines without *H. defensa* or other facultative symbionts. Letters above bars differentiate lines with statistically different rates of parasitism. Asterisks indicate significant differences between single *H. defensa* bearing lines vs. uninfected aphids of the same clone (UC) when only two lines were tested.

recombination within variants. For example, across three modules (including the virulence module), the APSE8 from B-strain isolate 5DH is most similar to APSE8 from E-strain isolate ZA17. But across most genes in Module 4, the 5DH APSE8 phage is more similar to APSE1 (Figure 1B). The expected phylogenetic discordance among Modules 1–3 versus Module 4 was further evidenced from our single APSE gene phylogenies (Figure S5).

Phage toxin type as major determinant of protective phenotype

To partition phage versus *H. defensa* effects on protective phenotypes, we leveraged (1) findings of distinct APSEs in otherwise highly similar *H. defensa*, and (2) distinct *H. defensa* strains carrying highly similar phage. In the first parasitism assay, we held aphid genotype constant (5DAB clone), examining successful parasitism rates across four isogenic sublines (Table 1). One subline was free of *H. defensa*, two sublines harboured B-strain *H. defensa* with different phages: ASPE8 encoding a *cdtB*$_2$ toxin; and APSE10 with a Shiga-like (*sltxA*$_2$) toxin. The fourth harboured E-strain *H. defensa* with APSE8/*cdtB*$_2$. Given that mortality outside of mummification did not differ significantly among sublines ($\bar{x} = 6.4$, range $4.5-8.1$; Analysis of Variance (ANOVA) $F_{3,33} = 1.9$, $p = 0.15$) we used the proportion of mummies/(mummies + surviving aphids) as our proxy for successful parasitism for consistency with past studies (e.g., Oliver et al., 2005). We found that all three *H. defensa*-bearing lines exhibited significantly reduced rates of successful parasitism after parasitism by *A. ervi* compared with *H. defensa*-free controls (Figure 2B; LRE (logistic regression equation, $Y = -1.41 + 0.65^{E+8} + 0.89^{B+8} + 1.56^{B+10}$; LRT $F_{E+8} = 19.3$ $p < 0.001$, $F_{B+8} = 38.3$, $p < 0.001$; $F_{B+10} = 124.2$ $p < 0.001$). This indicated effective symbiont defence in all examined lines.

Successful parasitism rates in the sublines harbouring APSE8 with identical *cdtB*$_2$ genes, were not

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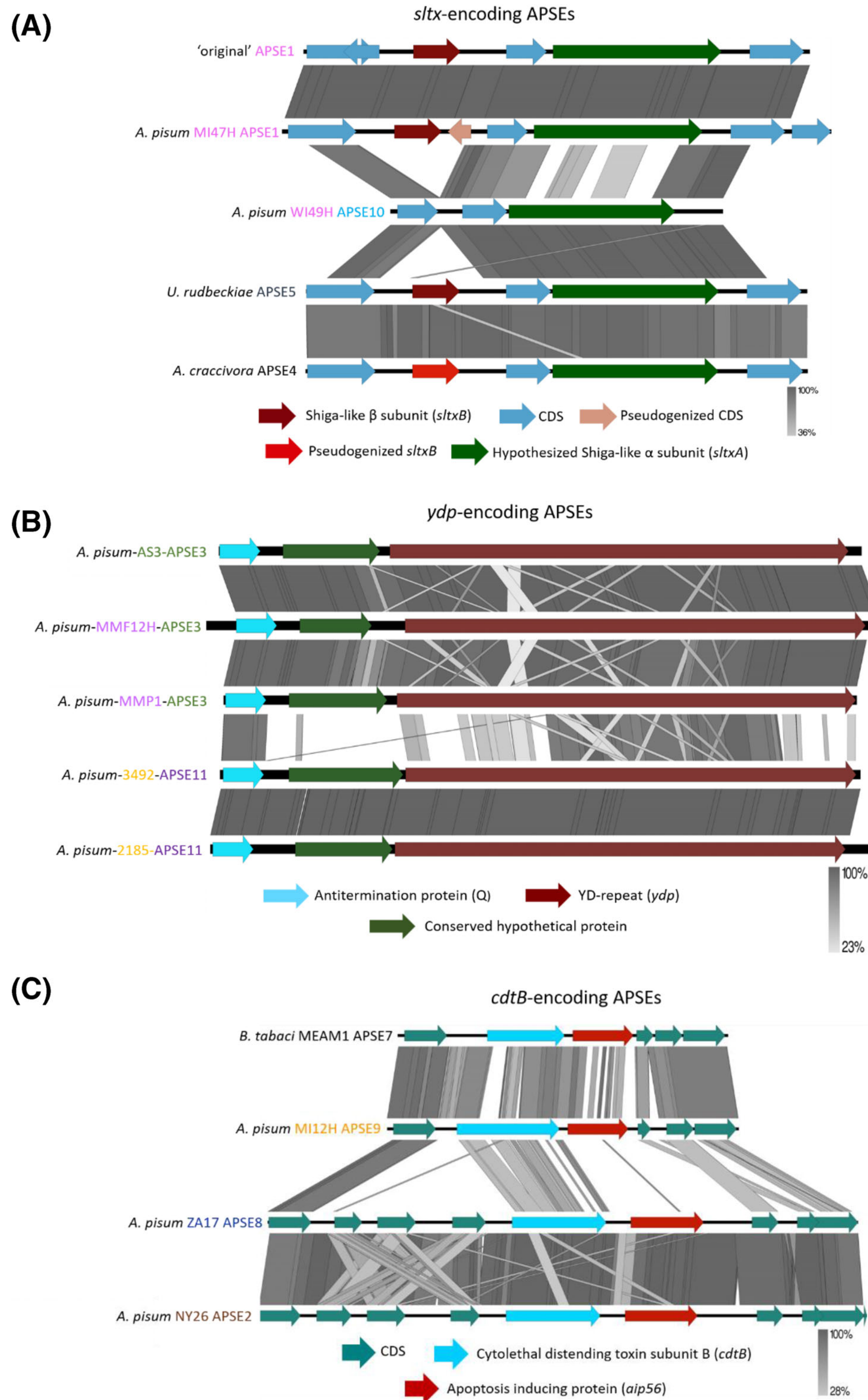

FIGURE 3 Variation in virulence modules for APSE variants encoding (A) *stlxA*, (B) *ydp* and (C) *cdtB*. The colour of the *H. defensa* isolate name (e.g., MI47H) matches the A–E clade designation in Figure 2 (pink, B-clade *H. defensa* strains). Similarly, the colour of the APSE variant name (e.g., pink, APSE1) matches the variant designation in Figure 2. CDS, coding sequence.

significantly different (Figure 2B; Tukey–Kramer HSD, $p = 0.91$), despite their occurrence in *H. defensa* isolates residing in different clades (B and E). In contrast, sublines with B-strain isolates harbouring different APSEs provided varying levels of anti-parasitoid protection. For instance, in aphid sublines with B-strain isolates harbouring APSE10/*sltxA*$_2$ (WI49H) the odds of successful parasitism decreased by 73% compared

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with those B-strain isolates carrying APSE8/*cdtB*$_2$ (5DH; Figure 2B: LRT $F_{B+10} = 12.6$, $p \leq 0.001$, Tukey–Kramer HSD, $p = 0.003$).

We also assessed a third B-clade *H. defensa* in the 5DAB genotype. This isolate carries an APSE3 with a toxin cassette that is distinct from those in the highly protective D-clade APSEs (Figure 3B; Oliver & Higashi, 2019). This isolate also conferred significant protection compared with the uninfected control (Figure 2C; *LRT* $F = 9.5$; $p = 0.002$). Since rates of successful parasitism did not vary between the two symbiont-free controls (both aphid genotype 5DAB) between assays (Figure 2B,C: LRT $F = 0.17$; $p = 0.68$), we conducted an analysis across all B-clade isolates finding that protection levels varied significantly with APSE variant (Figure 3B,C; ANOVA, $F_{3,40} = 39.6$ $p < 0.001$). A Tukey–Kramer HSD test showed that B-clade isolates with APSE10, for example, conferred significantly more protection than those with APSE8 ($p = 0.003$) or APSE3 ($p < 0.0001$).

The fourth B-clade *H. defensa* we examined harboured an APSE1 (MI47H) with a similar virulence module, and identical Shiga-like toxin alleles (*sltxA* analogue, *sltxB* homologue), to those of the reference APSE1 (Figure 3A; van der Wilk et al., 1999). This isolate of *H. defensa* conferred total protection (i.e., no mummies produced) while the antibiotic-cured control line MI47-AB was highly susceptible to parasitism (Figure 2D; LRT $F = 238.9$; $p < 0.0001$). Since this B-clade isolate produced no mummies (Figure 2D), and occurred in a different aphid genotype, we were unable to directly compare parasitism outcomes to the other B-clade isolates (Figure 2B,C) using a Tukey–Kramer test. However, given that this isolate conferred total protection, it appears very likely this APSE1-bearing isolate also differs in strength of protection despite sharing similar bacterial B-clade genotype (Figure 2D).

We next examined the protective phenotype of a C-strain *H. defensa*, MI12H, which carries an APSE9 with a virulence module that is most similar to APSE7 identified from *H. defensa* in the whitefly *B. tabaci* (Figure 2E; Boyd et al., 2021; Degnan & Moran, 2008a; Rouïl et al., 2020). This APSE9 encodes a *cdtB* allele that we label as *cdtB*$_3$—and is 59% similar at the AA level to the allele from APSE7, and shows ∼38% similarity to the *cdtB*$_1$ and *cdtB*$_2$ found, respectively, in APSE2 and 8. However, the amino acid residues required for DNase activity in pathogenic bacteria are conserved among *H. defensa*-encoded *cdtB* alleles (Figures 3 and S6; Lara-Tejero & Galán, 2000). Parasitism assays show that this C-clade/APSE9/*cdtB*$_3$ greatly reduces rates of successful parasitism by *A. ervi* compared with susceptible isogenic uninfected controls (AS3; Figure 2E; LR LRT $F = 303.9$, $p < 0.0001$). To generalize this result, we assayed a second C-clade *H. defensa* isolate (2184H) with an identical *cdtB*$_3$-encoding APSE9 but in a different aphid genotype. This isolate also conferred very high levels of protection relative to the isogenic, symbiont-free control (Figure 2E; LR LRT $F = 185.9$ $p < 0.0001$).

DISCUSSION

In animal-microbe symbioses, strain-level identity can be as important as species identity in determining outcomes of biotic interactions (Ballinger & Perlman, 2017; Dennis et al., 2017; Ginete & Goodrich-Blair, 2021; Martinez et al., 2017; Murfin et al., 2015; Oliver et al., 2005; Wu et al., 2022). In pea aphids, strain variation present in the anti-parasitoid symbiont *H. defensa*, correlates with variability in symbiont-mediated phenotypes and the maintenance of specific coinfections (Oliver et al., 2005; Peng et al., 2023; Smee et al., 2021; Weldon et al., 2013; Weldon et al., 2020, 2022), but the basis of functional variation is only partly understood. Here we used genomics and bioassays to better understand the role of strain-variation in generating observed variability in biotic interactions.

We conducted comparative genomics of independently derived isolates of *H. defensa* from B- ($N = 2$) and C-clade strains ($N = 1$) to complement previously completed genomes from isolates derived from A ($N = 2$), D ($N = 2$) and E ($N = 1$) strains (Chevignon et al., 2018; Degnan et al., 2009). Having one or more long-read genomes representing strains residing in all five common *H. defensa* clades allowed us first to generalize two previously observed patterns regarding gene content and organization (Chevignon et al., 2018). First, genes involved with housekeeping functions, metabolism, and nutrient uptake are nearly identical among isolates from different clades. For example, the newly considered B- and C-strain genomes were virtually identical in these respects compared with A-, B- and E-clade strains (e.g., Figure S1). Second, MGE content and genome architecture are also very similar for within-clade comparisons but differ across among-clade comparisons (Figures 1 and S3 and Tables S3–S8).

A clear exception to the pattern of similar MGE content in same-clade *H. defensa* isolates involved APSE prophages. While isolates from three *H. defensa* clades were closely associated with particular APSEs (clade D with APSE3, A with ASPE2, and E with ASPE8) those from the B- and C-clades were each associated with multiple APSE variants (Figure 2A). This indicates repeated lateral transfer of APSE into these lineages. In some instances, genomic data were consistent with the transfer of entire APSEs (e.g., APSE3 from MMF12H). In other cases, data supported the transfer and subsequent recombination with resident phages resulting in a new mosaic genotype. For example, the APSE variant in isolate 5DH has three modules similar to APSE8, but the fourth is most similar to APSE1. Both

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APSE1 and APSE8 regularly occur in B-clade *H. defensa* (Figure 2). We have identified uncommon instances in which two different *H. defensa* isolates are present in individual field-collected pea aphids (Lynn-Bell, 2021), providing ecological opportunities for phage recombination. However, it is also possible that module recombination occurred prior to their arrival in these strains. In addition to replacing or recombining with resident genotypes, APSEs also potentially move into phage-free *H. defensa*. These, however, appear rare in natural populations (Russell et al., 2013) and phage loss has been observed regularly only in D-clade strains (Oliver et al., 2009; Weldon et al., 2013), which showed no APSE variation in this study (Figure 2A). Studies with more resolution are needed to determine the extent and tempo of phage exchange in natural populations and effects on biotic interactions.

The presence of distinct APSEs in otherwise highly similar isolates of B-clade *H. defensa* allowed us to partition the contributions of *H. defensa*, APSE, and associated toxins to the anti-parasitoid phenotype. When holding *H. defensa* constant, we found that variation in the APSE virulence module best determined protection levels. For example, isolates of B- and E-clade *H. defensa*, each containing APSE8 with $CdtB_2$, conferred intermediate protection levels, while B-clade isolates with different APSEs/toxin variants conferred varying protection levels (Figure 2).

We also examined the anti-parasitoid phenotype of C-strain *H. defensa* for the first time finding that two independently derived isolates with *$cdtB_3$*-encoding APSE9 each conferred nearly complete protection against *A. ervi* wasps (Figure 2E). High protection was unexpected given several prior reports that *cdtB*-encoding APSE conferred only moderate protection (Doremus & Oliver, 2017; Martinez, Weldon, & Oliver, 2014; Oliver et al., 2005). For example, assayed A-, B-, and E-clade *H. defensa* with APSE2 or APSE8, with similar *cdtB* alleles (*$cdtB_1$ and $cdtB_2$*, respectively) confer intermediate levels of host protection (Oliver & Higashi, 2019). Similarly, we also observed variable protection by B-clade *H. defensa* with different *sltxA* alleles (Figure 2B,D), although these were separate assays and so results are not directly comparable. Further, B-clade *H. defensa* with APSE3 conferred relative low levels of protection (Figure 2C), which contrasts with numerous previously assayed D-clade APSE3s that were highly protective (Oliver et al., 2009). There is variation in the virulence modules (notably in the hypothetical conserved protein just upstream of *ydp*) between high and low protectors (Figure 3B) which may explain the observed variability in parasitoid outcomes. Other, not mutually exclusive reasons, may also be involved, including variation in wasp genotypes in their ability to overcome specific *H. defensa* pathogenicity factors. While this phenomenon has not been reported in pea aphids, it has been well demonstrated in *Lysiphlebus fabarum* parasitoids attacking *H. defensa*-infected black bean aphids (*Aphis fabae*; Dennis et al., 2017; Rouchet & Vorburger, 2012; Vorburger et al., 2009).

Together, these results are consistent with the specific toxin allele/s (rather than general toxin type or APSE variant) being the key determinant of protective phenotype. Interestingly, APSE-associated toxin homologues, including *cdtB* and *aip56* (Figure 3C) have been repeatedly horizontally transferred into the genomes of insects from at least five orders (Verster et al., 2019, 2023). In drosophilid fruit flies, laterally acquired toxins were recently shown to function in defence against parasitoids (Verster et al., 2023). Given that the hyperdiverse parasitoids are ubiquitous threats to insects and other arthropods (Forbes et al., 2018), these toxins may have exceptionally broad impacts on insect immunity through both defensive symbiosis and horizontal gene transfer (Oliver, 2023).

Since this is the first assay reporting the protective phenotype of APSE10-bearing *H. defensa*, we briefly discuss how this variant differs from other APSEs. First, typing loci from each of the three non-virulence modules shows this variant is distinct from previously named variants (Figure S5). The virulence module also differs from other *sltx*-encoding APSEs (Figure 3). In pathogenic bacteria with Shiga-toxins, a set of StxB subunits create tubular invaginations that facilitate entry of the active toxin StxA into target cells (Melton-Celsa, 2014). Other *sltx*-encoding APSEs have a protein (P7) with homology to StxB that is completely lacking in APSE10 (Figure 3A). That the *sltxB* gene is absent in this highly protective *H. defensa*/APSE (Figure 2) indicates it is not required for the protective phenotype in this interaction, and possibly more generally. Consistent with this, the *sltxB* gene is also pseudogenized in an APSE4-containing *H. defensa* isolate derived from *Aphis craccivora* that is protective against different parasitoid species (Asplen et al., 2014; Dykstra et al., 2014). Another protein (P9) in the cassette module was hypothesized to be a functional analog of the active subunit StxA (Figure 3A; Degnan & Moran, 2008a; van der Wilk et al., 1999). This *stlxA* gene is conserved across all *sltx*-encoding APSEs suggesting that it is the key component. The *stlxA* variant from APSE10 is very similar (94%–98% at the AA level) to those from APSE4 (protective; Asplen et al., 2014) and APSE5 (unknown protection), but less similar (46%) to those from APSE1 (protective; Figure 2). We also identified a novel phage variant, named APSE11, based on information from all four modules, that encodes a YDp toxin that is ∼63% similar at the AA level to the highly protective ASPE3s (Figures 4 and S4). However, we have not yet assayed *H. defensa* with APSE11 to assess its protective phenotype.

Combining past and present results involving similar bioassay protocols, we find that pea aphid clones

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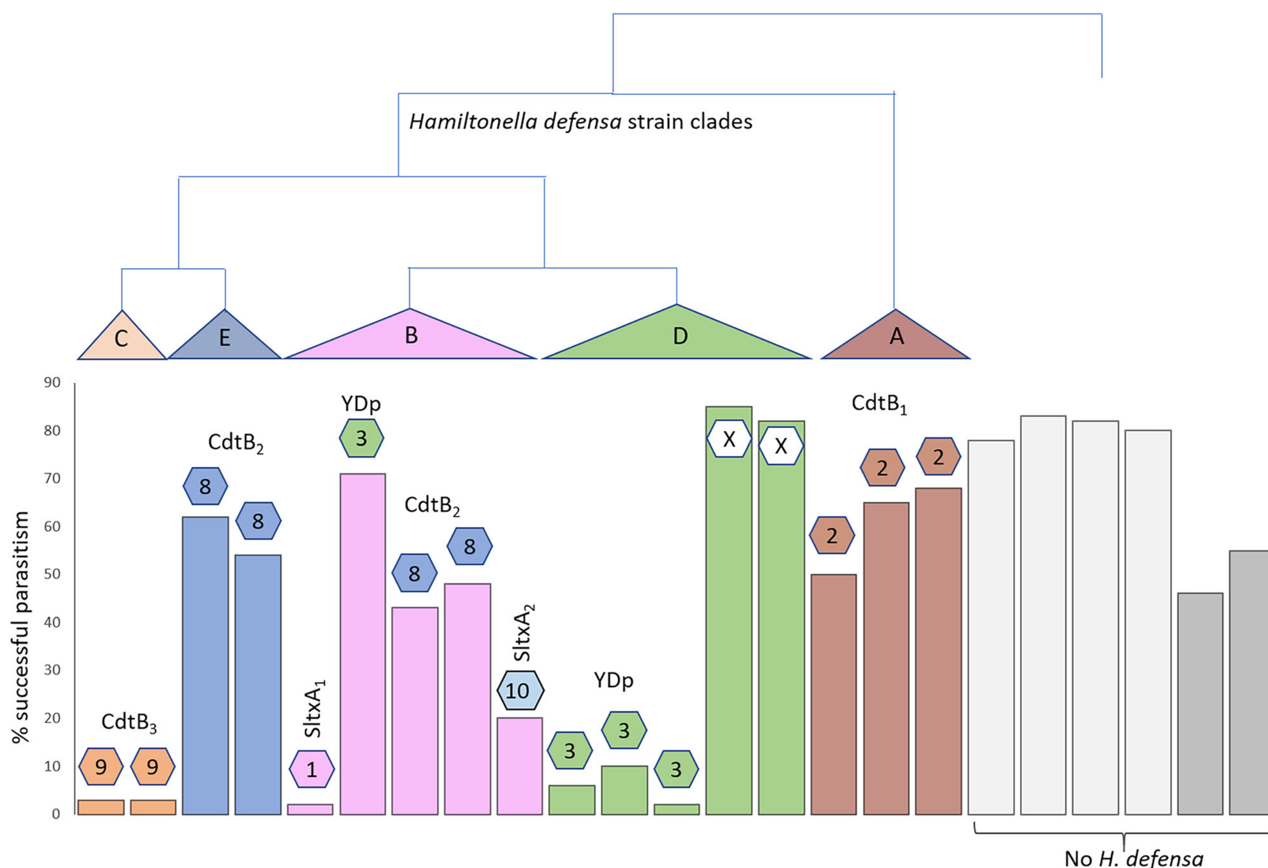

FIGURE 4 Summary figure showing the protective phenotype of *H. defensa* in relation to bacterial strain and phage/toxin variants. Coloured bars indicate bacterial strain clade (A–E) that match colour in phylogeny. Light grey columns represent typical controls lacking *H. defensa* although a few aphid genotypes conferring endogenous resistance are shown in dark grey (Martinez et al., 2018; Martinez, Ritter, et al., 2014; Martinez, Weldon, & Oliver, 2014). Hexagons above columns indicate APSE variant with '*X*' indicating 'no APSE' present. Toxin types (e.g., $CdtB_1$) are typed above columns/hexagons. *Y*-axis indicates % successful parasitism of wasp *Aphidius ervi* attacking aphids with a particular *H. defensa*/APSE/toxin variant relative to uninfected control. Results derived from parasitism assays in this study and prior assays using similar bioassay protocols (Doremus et al., 2018; Higashi et al., 2020; Martinez et al., 2018; Oliver et al., 2009).

lacking *H. defensa* are mostly susceptible to the wasp *A. ervi*, although a few aphid genotypes encode endogenous resistance (Martinez, Ritter, et al., 2014). In contrast, H. *defensa* isolates derived from five bacterial clades (A–E), and associated with six APSE variants (APSE1, 2, 3, 8, 9, and 10) and six total toxin/cassette types (3 *cdtB*, 2 *sltx*, and 2 *ydp*) all confer significant, but variable protection against this aphid's most common parasitoid (Figure 4; Doremus et al., 2018; Higashi et al., 2020; Martinez et al., 2018; Oliver et al., 2009; Oliver & Higashi, 2019). Considering that other biotypes of pea aphids, and other aphid species, are associated with distinct strains of *H. defensa* and APSE further indicates the potential for substantial diversity in bioactive elements across this defensive symbiosis (Cayetano et al., 2015; Degnan & Moran, 2008a, 2008b; Dykstra et al., 2014; Henry et al., 2013, 2022; Wu et al., 2022). In turn, diversity in symbiont defences may drive parasitoid diversity and specialization, and resulting in co-evolutionary interactions (Hafer & Vorburger, 2019; Vorburger, 2022; Vorburger & Perlman, 2018). Strain variation is likely to impact infection dynamics in natural populations with practical implications for the biological control of pests and symbiont-mediated phenotypes in a rapidly changing climate (Desneux et al., 2018; Higashi et al., 2020; Ives et al., 2020; Smith et al., 2015; Vorburger, 2018).

We also examined variability in MGE content in efforts to understand the basis of B-clade *H. defensa* strongly associating with *F. symbiotica*. We found no support for metabolic cooperation between *H. defensa* and *F. symbiotica* in this study (Figure S1) or a previous one (Peng et al., 2023). Prior studies also found no net fitness benefits to aphids that carried both *F. symbiotica* and B-clade *H. defensa* compared with those carrying *H. defensa* alone (Doremus et al., 2018; Doremus & Oliver, 2017)—although such phenotypes have been reported for some European strains of the same symbiont species (Heyworth & Ferrari, 2015; Smee et al., 2021). However, because aphids carrying only *F. symbiotica* exhibited large reductions in fitness

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that were partially mitigated in aphids also harbouring B-clade *H. defensa* (Doremus & Oliver, 2017), we predicted in this article that mobilome-encoded factors that mediate within-aphid competition between symbionts may have roles in structuring the heritable microbiome. For example, factors encoded in one symbiont species may reduce the abundance or virulence of a second species. We identified numerous among strain differences in MGE content (Tables S6–S9), including a bacteriocin-encoding plasmid that is present in almost all most B-clade *H. defensa*, but was not detected in strains residing in other clades (Table S9). Experimental work is needed to determine whether this plasmid influences the propensity of B-clade strains to more readily form coinfections. In conclusion, our results indicate that strain-level variation in MGE content has important consequences for the defensive phenotype of *H. defensa*, and possibly plays a role in the maintenance of specific co-infections.

AUTHOR CONTRIBUTIONS

Vilas Patel: Data curation (lead); formal analysis (equal); investigation (equal); methodology (equal); supervision (supporting); visualization (equal); writing—original draft (supporting); writing—review and editing (equal). **Nicole Lynn-Bell:** Data curation (supporting); formal analysis (equal); investigation (equal); methodology (equal); visualization (supporting); writing—review and editing (supporting). **Germain Chevignon:** Formal analysis (supporting); writing—review and editing (supporting). **Roy A. Kucuk:** Investigation (supporting); writing—review and editing (supporting). **Clesson H. V. Higashi:** Methodology (supporting); writing – review and editing (supporting). **Melissa Carpenter:** Methodology (supporting); writing—review and editing (supporting). **Jacob A. Russell:** Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal); writing—original draft (equal); writing—review and editing (equal). **Kerry Oliver:** Conceptualization (equal); formal analysis (supporting); funding acquisition (equal); project administration (equal); supervision (equal); visualization (supporting); writing—original draft (lead); writing—review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data generated in association with this study are either publicly available where noted or found in Table S10 published online.

ORCID

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REFERENCES

Angalet, G.W. & Fuester, R. (1977) The *Aphidius* parasites of the pea aphid *Acyrthosiphon pisum* in the rastern half of the United States. *Annals of the Entomological Society of America*, 70, 87–96.

Arndt, D., Grant, J.R., Marcu, A., Sajed, T., Pon, A., Liang, Y.J. et al. (2016) PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Research*, 44, W16–W21.

Asplen, M.K., Bano, N., Brady, C.M., Desneux, N., Hopper, K.R., Malouines, C. et al. (2014) Specialisation of bacterial endosymbionts that protect aphids from parasitoids. *Ecological Entomology*, 39, 736–739.

Ballinger, M.J. & Perlman, S.J. (2017) Generality of toxins in defensive symbiosis: ribosome-inactivating proteins and defense against parasitic wasps in *Drosophila*. *PLoS Pathogens*, 13, e1006431.

Ballinger, M.J. & Perlman, S.J. (2019) The defensive *Spiroplasma*. *Current Opinion in Insect Science*, 32, 36–41.

Boyd, B.M., Chevignon, G., Patel, V., Oliver, K.M. & Strand, M.R. (2021) Evolutionary genomics of APSE: a tailed phage that lysogenically converts the bacterium *Hamiltonella defensa* into a heritable protective symbiont of aphids. *Virology Journal*, 18, 219.

Brandt, J.W., Chevignon, G., Oliver, K.M. & Strand, M.R. (2017) Culture of an aphid heritable symbiont demonstrates its direct role in defence against parasitoids. *Proceedings of the Royal Society B: Biological Sciences*, 284, 0171925.

Brettin, T., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Olsen, G.J. et al. (2015) RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Scientific Reports*, 5, 8365.

Breusing, C., Genetti, M., Russell, S.L., Corbett-Detig, R.B. & Beinart, R.A. (2022) Horizontal transmission enables flexible associations with locally adapted symbiont strains in deep-sea hydrothermal vent symbioses. *Proceedings of the National Academy of Sciences of the United States of America*, 119, e2115608119.

Canchaya, C., Fournous, G., Chibani-Chennoufi, S., Dillmann, M.L. & Brüssow, H. (2003) Phage as agents of lateral gene transfer. *Current Opinion in Microbiology*, 6, 417–424.

Cayetano, L., Rothacher, L., Simon, J.C. & Vorburger, C. (2015) Cheaper is not always worse: strongly protective isolates of a defensive symbiont are less costly to the aphid host. *Proceedings of the Royal Society B: Biological Sciences*, 282, 20142333.

Chafee, M.E., Funk, D.J., Harrison, R.G. & Bordenstein, S.R. (2010) Lateral phage transfer in obligate intracellular bacteria (Wolbachia): verification from natural populations. *Molecular Biology and Evolution*, 27, 501–505.

Chevignon, G., Boyd, B.M., Brandt, J.W., Oliver, K.M. & Strand, M.R. (2018) Culture-facilitated comparative genomics of the facultative symbiont *Hamiltonella defensa*. *Genome Biology and Evolution*, 10, 786–802.

Dawid, S., Roche, A.M. & Weiser, J.N. (2007) The blp bacteriocins of Streptococcus pneumoniae mediate intraspecies competition both in vitro and in vivo. *Infection and Immunity*, 75, 443–451.

14622920, 2023, 12, Downloaded from https://ami-journals.onlinelibrary.wiley.com/doi/10.1111/1462-2920.16520 by IFREMER Centre Bretagne BLP, Wiley Online Library on [03/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Degnan, P.H., Leonardo, T.E., Cass, B.N., Hurwitz, B., Stern, D., Gibbs, R.A. et al. (2010) Dynamics of genome evolution in facultative symbionts of aphids. *Environmental Microbiology*, 12, 2060–2069.

Degnan, P.H. & Moran, N.A. (2008a) Diverse phage-encoded toxins in a protective insect endosymbiont. *Applied and Environmental Microbiology*, 74, 6782–6791.

Degnan, P.H. & Moran, N.A. (2008b) Evolutionary genetics of a defensive facultative symbiont of insects: exchange of toxin-encoding bacteriophage. *Molecular Ecology*, 17, 916–929.

Degnan, P.H., Yu, Y., Sisneros, N., Wing, R.A. & Moran, N.A. (2009) *Hamiltonella defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 9063–9068.

Dennis, A.B., Patel, V., Oliver, K.M. & Vorburger, C. (2017) Parasitoid gene expression changes after adaptation to symbiont-protected hosts. *Evolution*, 71, 2599–2617.

Desneux, N., Asplen, M.K., Brady, C.M., Heimpel, G.E., Hopper, K.R., Luo, C. et al. (2018) Intraspecific variation in facultative symbiont infection among native and exotic pest populations: potential implications for biological control. *Biological Control*, 116, 27–35.

Doremus, M.R. & Oliver, K.M. (2017) Aphid heritable symbiont exploits defensive mutualism. *Applied and Environmental Microbiology*, 83, e03276-16.

Doremus, M.R., Smith, A.H., Kim, K.L., Holder, A.J., Russell, J.A. & Oliver, K.M. (2018) Breakdown of a defensive symbiosis, but not endogenous defences, at elevated temperatures. *Molecular Ecology*, 27, 2138–2151.

Dykstra, H.R., Weldon, S.R., Martinez, A.J., White, J.A., Hopper, K.R., Heimpel, G.E. et al. (2014) Factors limiting the spread of the protective symbiont Hamiltonella defensa in Aphis craccivora aphids. *Applied and Environmental Microbiology*, 80, 5818–5827.

Ferrari, J., West, J.A., Via, S. & Godfray, H.C.J. (2012) Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. *Evolution*, 66, 375–390.

Forbes, A.A., Bagley, R.K., Beer, M.A., Hippee, A.C. & Widmayer, H.A. (2018) Quantifying the unquantifiable: why Hymenoptera, not coleoptera, is the most speciose animal order. *BMC Ecology*, 18, 21.

Ginete, D.R. & Goodrich-Blair, H. (2021) From binary model systems to the human microbiome: factors that drive strain specificity in host-symbiont associations. *Frontiers in Ecology and Evolution*, 9, 614197.

Guo, J.Q., Hatt, S., He, K.L., Chen, J.L., Francis, F. & Wang, Z.Y. (2017) Nine facultative endosymbionts in aphids. A review. *Journal of Asia-Pacific Entomology*, 20, 794–801.

Hafer, N. & Vorburger, C. (2019) Diversity begets diversity: do parasites promote variation in protective symbionts? *Current Opinion in Insect Science*, 32, 8–14.

Hedges, L.M., Brownlie, J.C., O'Neill, S.L. & Johnson, K.N. (2008) *Wolbachia* and virus protection in insects. *Science*, 322, 702.

Henry, L.M., Peccoud, J., Simon, J.C., Hadfield, J.D., Maiden, M.J., Ferrari, J. et al. (2013) Horizontally transmitted symbionts and host colonization of ecological niches. *Current Biology*, 23, 1713–1717.

Henry, Y., Brechbühler, E. & Vorburger, C. (2022) Gated communities: inter- and intraspecific diversity of endosymbionts across four sympatric aphid species. *Frontiers in Ecology and Evolution*, 10, 816184.

Heyworth, E.R. & Ferrari, J. (2015) A facultative endosymbiont in aphids can provide diverse ecological benefits. *Journal of Evolutionary Biology*, 28, 1753–1760.

Heyworth, E.R., Smee, M.R. & Ferrari, J. (2020) Aphid facultative symbionts aid recovery of their obligate symbiont and their host after heat stress. *Frontiers in Ecology and Evolution*, 8, 56.

Higashi, C.H.V., Barton, B.T. & Oliver, K.M. (2020) Warmer nights offer no respite for a defensive mutualism. *Journal of Animal Ecology*, 89, 1895–1905.

Higashi, C.H.V., Nichols, W.L., Chevignon, G., Patel, V., Allison, S.E., Kim, K.L. et al. (2023) An aphid symbiont confers protection against a specialized RNA virus, another increases vulnerability to the same pathogen. *Molecular Ecology*, 32, 936–950.

Hoang, D.T., Chernomor, O., von Haeseler, A., Minh, B.Q. & Vinh, L.S. (2018) UFBoot2: improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution*, 35, 518–522.

Ives, A.R., Barton, B.T., Penczykowski, R.M., Harmon, J.P., Kim, K.L., Oliver, K. et al. (2020) Self-perpetuating ecological-evolutionary dynamics in an agricultural host-parasite system. *Nature Ecology & Evolution*, 4, 702–711.

Kalkatawi, M., Alam, I. & Bajic, V.B. (2015) BEACON: automated tool for bacterial GEnome annotation ComparisON. *BMC Genomics*, 16, 616.

Kaltenpoth, M. & Engl, T. (2014) Defensive microbial symbionts in Hymenoptera. *Functional Ecology*, 28, 315–327.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A. & Jermiin, L.S. (2017) ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods*, 14, 587–589.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M. & Tanabe, M. (2016) KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Research*, 44, D457–D462.

Kent, B.N., Funkhouser, L.J., Setia, S. & Bordenstein, S.R. (2011) Evolutionary genomics of a temperate bacteriophage in an obligate intracellular bacteria (*Wolbachia*). *PLoS One*, 6, e24984.

Kent, B.N., Salichos, L., Gibbons, J.G., Rokas, A., Newton, I.L.G., Clark, M.E. et al. (2011) Complete bacteriophage transfer in a bacterial endosymbiont (*Wolbachia*) determined by targeted genome capture. *Genome Biology and Evolution*, 3, 209–218.

Klein, T.A., Ahmad, S. & Whitney, J.C. (2020) Contact-dependent interbacterial antagonism mediated by protein secretion machines. *Trends in Microbiology*, 28, 387–400.

Kreimer, A., Doron-Faigenboim, A., Borenstein, E. & Freilich, S. (2012) NetCmpt: a network-based tool for calculating the metabolic competition between bacterial species. *Bioinformatics*, 28, 2195–2197.

Lagesen, K., Hallin, P., Rødland, E.A., Staerfeldt, H.H., Rognes, T. & Ussery, D.W. (2007) RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Research*, 35, 3100–3108.

Lara-Tejero, M. & Galán, J.E. (2000) A bacterial toxin that controlsc cell cycle progression as a deoxyribonuclease I-like protein. *Science*, 290, 354–357.

Lo, W.-S. & Kuo, C.-H. (2017) Horizontal acquisition and transcriptional integration of novel genes in mosquito-associated *Spiroplasma*. *Genome Biology and Evolution*, 9, 3246–3259.

Lukasik, P., van Asch, M., Guo, H.F., Ferrari, J. & Godfray, H.C.J. (2013) Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecology Letters*, 16, 214–218.

Lynn-Bell, N. (2021) Bacteriophage roles in the diversity and horizontal transfer of protective functions in the heritable bacterial symbiont, *Hamiltonella defensa*. (PhD dissertation; University of Georgia).

Lynn-Bell, N.L., Strand, M.R. & Oliver, K.M. (2019) Bacteriophage acquisition restores protective mutualism. *Microbiology*, 165, 985–989.

Markowitz, V.M., Chen, I.M.A., Palaniappan, K., Chu, K., Szeto, E., Grechkin, Y. et al. (2012) IMG: the integrated microbial genomes database and comparative analysis system. *Nucleic Acids Research*, 40, D115–D122.

Martinez, A.J., Doremus, M.R., Kraft, L.J., Kim, K.L. & Oliver, K.M. (2018) Multi-modal defences in aphids offer redundant protection and increased costs likely impeding a protective mutualism. *Journal of Animal Ecology*, 87, 464–477.

Martinez, A.J., Ritter, S.G., Doremus, M.R., Russell, J.A. & Oliver, K.M. (2014) Aphid-encoded variability in susceptibility to a parasitoid. *BMC Evolutionary Biology*, 14, 127.

14622920, 2023, 12, Downloaded from https://ami-journals.onlinelibrary.wiley.com/doi/10.1111/1462-2920.16520 by IFREMER Centre Bretagne BLP, Wiley Online Library on [03/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Martinez, A.J., Weldon, S.R. & Oliver, K.M. (2014) Effects of parasitism on aphid nutritional and protective symbioses. *Molecular Ecology*, 23, 1594–1607.

Martinez, J., Tolosana, I., Ok, S., Smith, S., Snoeck, K., Day, J.P. et al. (2017) Symbiont strain is the main determinant of variation in *Wolbachia*-mediated protection against viruses across *Drosophila* species. *Molecular Ecology*, 26, 4072–4084.

McLean, A.H.C. & Godfray, H.C.J. (2015) Evidence for specificity in symbiont-conferred protection against parasitoids. *Proceedings of the Royal Society B: Biological Sciences*, 282, 20150977.

McLean, A.H.C., Hrcek, J., Parker, B.J., Mathe-Hubert, H., Kaech, H., Paine, C. et al. (2020) Multiple phenotypes conferred by a single insect symbiont are independent. *Proceedings of the Royal Society B: Biological Sciences*, 287, 20200562.

Melton-Celsa, A.R. (2014) Shiga toxin (Stx) classification, structure, and function. *Microbiology Spectrum*, 2, 2–4.

Meseguer, A.S., Manzano-Marin, A., Coeur D'Acier, A., Clamens, A.L., Godefroid, M. & Jousselin, E. (2017) *Buchnera* has changed flatmate but the repeated replacement of co-obligate symbionts is not associated with the ecological expansions of their aphid hosts. *Molecular Ecology*, 26, 2363–2378.

Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., von Haeseler, A. et al. (2020) IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution*, 37, 1530–1534.

Moran, N.A., Degnan, P.H., Santos, S.R., Dunbar, H.E. & Ochman, H. (2005) The players in a mutualistic symbiosis: insects, bacteria, viruses, and virulence genes. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 16919–16926.

Murfin, K.E., Lee, M.-M., Klassen, J.L., McDonald, B.R., Larget, B., Forst, S. et al. (2015) *Xenorhabdus bovienii* strain diversity impacts coevolution and symbiotic maintenance with *Steinernema* spp. nematode hosts. *MBio*, 6, e00076-00015.

Niepoth, N., Ellers, J. & Henry, L.M. (2018) Symbiont interactions with non-native hosts limit the formation of new symbioses. *BMC Evolutionary Biology*, 18, 27.

Oliver, K.M. (2023) Flies co-opt bacterial toxins for use in defense against parasitoids. *Proceedings of the National Academy of Sciences of the United States of America*, 120, e2304493120.

Oliver, K.M., Degnan, P.H., Burke, G.R. & Moran, N.A. (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annual Review of Entomology*, 55, 247–266.

Oliver, K.M., Degnan, P.H., Hunter, M.S. & Moran, N.A. (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science*, 325, 992–994.

Oliver, K.M. & Higashi, C.H.V. (2019) Variations on a protective theme: Hamiltonella defensa infections in aphids variably impact parasitoid success. *Current Opinion in Insect Science*, 32, 1–7.

Oliver, K.M. & Moran, N.A. (2009) Defensive symbionts in aphids and other insects. In: White, J.F. & Torres, M.S. (Eds.) *Defensive mutualism in microbial symbiosis*. Boca Raton: CRC Press, pp. 129–147.

Oliver, K.M., Moran, N.A. & Hunter, M.S. (2005) Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 12795–12800.

Oliver, K.M., Noge, K., Huang, E.M., Campos, J.M., Becerra, J.X. & Hunter, M.S. (2012) Parasitic wasp responses to symbiont-based defense in aphids. *BMC Biology*, 10, 11.

Oliver, K.M. & Perlman, S.J. (2020) Toxin-mediated protection against natural enemies by insect defensive symbionts. In: Oliver, K.M. & Russell, J.A. (Eds.) *Mechanisms underlying microbial Symbiosis*. London: Elsevier, pp. 277–316.

Oliver, K.M., Russell, J.A., Moran, N.A. & Hunter, M.S. (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 1803–1807.

Patel, V., Chevignon, G., Manzano-Marin, A., Brandt, J.W., Strand, M.R., Russell, J.A. et al. (2019) Cultivation-assisted genome of Candidatus *Fukatsuia symbiotica*; the enigmatic "X-type" symbiont of aphids. *Genome Biology and Evolution*, 11, 3510–3522.

Peccoud, J., Simon, J.C., McLaughlin, H.J. & Moran, N.A. (2009) Post-Pleistocene radiation of the pea aphid complex revealed by rapidly evolving endosymbionts. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 16315–16320.

Peng, L.Y., Hoban, J., Jonah, J., Smith, A.H., Carpenter, M., Marcelis, T. et al. (2023) Cryptic community structure and metabolic interactions among the heritable facultative symbionts of the pea aphid. *Journal of Evolutionary Biology* in revision. https://doi.org/10.1111/jeb.14216

Pietri, J.E., DeBruhl, H. & Sullivan, W. (2016) The rich somatic life of *Wolbachia*. *MicrobiologyOpen*, 5, 923–936.

Rodriguiz-R, L.M. & Konstantinidis, T.K. (2016) The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. *Peer J Preprints*, 4, e1900v1. Available from: https://doi.org/10.7287/peerj.preprints.1900v1

Ross, M.G., Russ, C., Costello, M., Hollinger, A., Lennon, N.J., Hegarty, R. et al. (2013) Characterizing and measuring bias in sequence data. *Genome Biology*, 14, R51.

Rouchet, R. & Vorburger, C. (2012) Strong specificity in the interaction between parasitoids and symbiont-protected hosts. *Journal of Evolutionary Biology*, 25, 2369–2375.

Rouïl, J., Jousselin, E., Coeur d'acier, A., Cruaud, C. & Manzano-Marín, A. (2020) The protector within: comparative genomics of APSE phages across sphids teveals tampant tecombination and fiverse toxin arsenals. *Genome Biology and Evolution*, 12, 878–889.

Russell, J.A., Latorre, A., Sabater-Muñoz, B., Moya, A. & Moran, N.A. (2003) Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Molecular Ecology*, 12, 1061–1075.

Russell, J.A. & Moran, N.A. (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proceedings of the Royal Society B: Biological Sciences*, 273, 603–610.

Russell, J.A., Weldon, S., Smith, A.H., Kim, K.L., Hu, Y., Lukasik, P. et al. (2013) Uncovering symbiont-driven genetic diversity across north American pea aphids. *Molecular Ecology*, 22, 2045–2059.

Scarborough, C.L., Ferrari, J. & Godfray, H.C.J. (2005) Aphid protected from pathogen by endosymbiont. *Science*, 310, 1781.

Schattner, P., Brooks, A.N. & Lowe, T.M. (2005) The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. *Nucleic Acids Research*, 33, W686–W689.

Schmid, M., Sieber, R., Zimmermann, Y.S. & Vorburger, C. (2012) Development, specificity and sublethal effects of symbiont-conferred resistance to parasitoids in aphids. *Functional Ecology*, 26, 207–215.

Scholz, M., Albanese, D., Tuohy, K., Donati, C., Segata, N. & Rota-Stabelli, O. (2020) Large scale genome reconstructions illuminate *Wolbachia* evolution. *Nature Communications*, 11, 5235.

Siguier, P., Perochon, J., Lestrade, L., Mahillon, J. & Chandler, M. (2006) ISfinder: the reference Centre for bacterial insertion sequences. *Nucleic Acids Research*, 34, D32–D36.

Smee, M.R., Raines, S.A. & Ferrari, J. (2021) Genetic identity and genotype x genotype interactions between symbionts outweigh species level effects in an insect microbiome. *ISME Journal*, 15, 2537–2546.

Smith, A.H., Lukasik, P., O'Connor, M.P., Lee, A., Mayo, G., Drott, M.T. et al. (2015) Patterns, causes and consequences of defensive microbiome dynamics across multiple scales. *Molecular Ecology*, 24, 1135–1149.

14622920, 2023, 12, Downloaded from https://ami-journals.onlinelibrary.wiley.com/doi/10.1111/1462-2920.16520 by IFREMER Centre Bretagne BLP, Wiley Online Library on [03/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Smith, A.H., O'Connor, M.P., Deal, B., Kotzer, C., Lee, A., Wagner, B. et al. (2021) Does getting defensive get you anywhere?-seasonal balancing selection, temperature, and parasitoids shape real-world, protective endosymbiont dynamics in the pea aphid. *Molecular Ecology*, 30, 2449–2472.

Speare, L., Cecere, A.G., Guckes, K.R., Smith, S., Wollenberg, M.S., Mandel, M.J. et al. (2018) Bacterial symbionts use a type VI secretion system to eliminate competitors in their natural host. *Proceedings of the National Academy of Sciences of the United States of America*, 115, E8528–E8537.

Sullivan, M.J., Petty, N.K. & Beatson, S.A. (2011) Easyfig: a genome comparison visualizer. *Bioinformatics*, 27, 1009–1010.

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E.P., Zaslavsky, L. et al. (2016) NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Research*, 44, 6614–6624.

Thappeta, K.R.V., Ciezki, K., Morales-Soto, N., Wesener, S., Goodrich-Blair, H., Stock, S.P. et al. (2020) R-type bacteriocins of *Xenorhabdus bovienii* determine the outcome of interspecies competition in a natural host environment. *Microbiology*, 166, 1074–1087.

van der Wilk, F., Dullemans, A.M., Verbeek, M. & van den Heuvel, J. (1999) Isolation and characterization of APSE-1, a bacteriophage infecting the secondary endosymbiont of *Acyrthosiphon pisum*. *Virology*, 262, 104–113.

Varani, A.M., Siguier, P., Gourbeyre, E., Charneau, V. & Chandler, M. (2011) ISsaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. *Genome Biology*, 12, R30.

Verster, K.I., Cinege, G., Lipinkszki, Z., Magyar, L.B., Kurucz, E., Tarnopol, R.L. et al. (2023) Evolution of insect innate immunity through domestication of bacterial toxins. *National Academy of Sciences of the United States of America*, 120, e2218334120.

Verster, K.I., Wisecaver, J.H., Karageorgi, M., Duncan, R.P., Gloss, A.D., Armstrong, E.E. et al. (2019) Horizontal transfer of bacterial Cytolethal distending toxin B genes to insects. *Molecular Biology and Evolution*, 36, 2105–2110.

Vorburger, C. (2014) The evolutionary ecology of symbiont-conferred resistance to parasitoids in aphids. *Insect Science*, 21, 251–264.

Vorburger, C. (2018) Symbiont-conferred resistance to parasitoids in aphids – challenges for biological control. *Biological Control*, 116, 17–26.

Vorburger, C. (2022) Defensive symbionts and the evolution of parasitoid host specialization. *Annual Review of Entomology*, 67, 329–346.

Vorburger, C. & Perlman, S.J. (2018) The role of defensive symbionts in host–parasite coevolution. *Biological Reviews*, 93, 1747–1764.

Vorburger, C., Sandrock, C., Gouskov, A., Castañeda, L.E. & Ferrari, J. (2009) Genotypic variation and the role of defensive endosymbionts in an all-parthenogenetic host–parasitoid interaction. *Evolution*, 63, 1439–1450.

Weldon, S.R., Russell, J.A. & Oliver, K.M. (2020) More is not always better: coinfections with defensive symbionts generate highly variable outcomes. *Applied and Environmental Microbiology*, 86, e02537-19.

Weldon, S.R., Strand, M.R. & Oliver, K.M. (2013) Phage loss and the breakdown of a defensive symbiosis in aphids. *Proceedings of the Royal Society B: Biological Sciences*, 280, 20122103.

Wu, T., Monnin, D., Lee, R.A.R. & Henry, L.M. (2022) Local adaptation to hosts and parasitoids shape *Hamiltonella defensa* genotypes across aphid species. *Proceedings of the Royal Society B: Biological Sciences*, 289, 20221269.

Zytynska, S.E. & Weisser, W.W. (2016) The natural occurrence of secondary bacterial symbionts in aphids. *Ecological Entomology*, 41, 13–26.

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