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Culture of an aphid heritable symbiont demonstrates its direct role in defence against parasitoids

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Heritable symbionts are common in insects with many contributing to host defence. *Hamiltonella defensa* is a facultative, bacterial symbiont of the pea aphid, *Acyrtosiphon pisum* that provides protection against the endoparasitoid wasp *Aphidius ervi*. Protection levels vary among strains of *H. defensa* that are differentially infected by bacteriophages named APSEs. By contrast, little is known about mechanism(s) of resistance owing to the intractability of host-restricted microbes for functional study. Here, we developed methods for culturing strains of *H. defensa* that varied in the presence and type of APSE. Most *H. defensa* strains proliferated at 27°C in co-cultures with the TN5 cell line or as pure cultures with no insect cells. The strain infected by APSE3, which provides high levels of protection *in vivo*, produced a soluble factor(s) that disabled development of *A. ervi* embryos independent of any aphid factors. Experimental transfer of APSE3 also conferred the ability to disable *A. ervi* development to a phage-free strain of *H. defensa*. Altogether, these results provide a critical foundation for characterizing symbiont-derived factor(s) involved in host protection and other functions. Our results also demonstrate that phage-mediated transfer of traits provides a mechanism for innovation in host restricted symbionts.

1. Introduction

Insects, including species of medical and agricultural importance, frequently host maternally transmitted bacterial symbionts [1]. By providing novel genes that expand the functional repertoire of the host insect, heritable symbionts often play key roles in mediating host ecology [2,3]. Phloem-feeding aphids (Hemiptera: Aphidoidea), for example, depend on the obligate (=primary) symbiont *Buchnera aphidicola*, which lives in specialized cells called bacteriocytes and supplies nutrients deficient in the aphid diet [4]. Aphids also commonly host one or more species of facultative (=secondary) symbionts that can live either extracellularly in the hemocoel or intracellularly in bacteriocytes or neighbouring sheath cells [5,6]. Facultative symbionts provide several conditional benefits to hosts including resistance to parasitoid wasps and pathogens, protection against thermal stress and facilitation of host plant usage [7–16]. Some facultative symbionts are also infected by bacteriophages [17–19] that enhance host fitness by provisioning novel traits and altering within-host bacterial abundance [18,20,21].

The γ -proteobacterium *Hamiltonella defensa* is among the most common aphid facultative symbionts, occurring in 34% of sampled species [22,23]. *Hamiltonella defensa* confers protection against parasitoids in at least three aphid species, including the pea aphid, *Acyrtosiphon pisum* [8,14,24]. Field and laboratory surveys identify multiple strains of *H. defensa* in *A. pisum* with most persistently infected by a podovirus-like bacteriophage named APSE [25–27]. Four APSE variants (APSE1, 2, 3 and 8) are currently known. The viral genome of each shares strong conservation in regulatory and structural genes, but varies in a 3.7–5.8 kb domain containing predicted eukaryotic-like toxin genes with hypothesized functions in parasitoid defence [18,19,28,29]. A major mortality agent of *A. pisum* is the endoparasitoid wasp *Aphidius ervi* [30–33]. In examined strains,

H. defensa confers protection against this wasp only when infected with APSE [34]. In APSE3-associated strains, the spontaneous loss of phage eliminates protection against *A. ervi* and increases titres of *H. defensa*, which adversely affect aphid fitness [20,21]. Protection levels against *A. ervi* and the timing of wasp mortality in aphids differ among *H. defensa* strains and APSE variants [20,24,29,35–37]. Recent results indicate that some genotypes of *A. pisum* exhibit resistance towards *A. ervi* in the absence of *H. defensa* or other facultative symbionts [38]. Endogenous and symbiont defences also target only specific parasitoid species [29,39]. These findings collectively suggest *A. pisum* defence against parasitoids is multifactorial with APSE-encoded toxins or other gene products interacting additively or synergistically with factors produced by aphids.

Asexual reproduction and the ability to manipulate symbiont infections make aphids excellent models for studying the phenotypic effects of facultative symbionts [6,7]. By contrast, *in vivo* studies have provided few insights into the mechanisms underlying symbiont-mediated phenotypes. The ability to culture normally host-restricted symbionts and their bacteriophages outside of aphids and other insects would open new avenues for studying function. While most heritable symbionts of insects remain unculturable, a few species have been propagated in co-cultures with insect cells [40–44] or as pure cultures [43,45]. The approximately 2.1 Mb genome of *H. defensa* is significantly smaller than its closest free-living relatives and lacks a number of genes with metabolic functions [46]. Nonetheless, Darby *et al.* [40] reported that one strain of *H. defensa* (called T type in this study) from black bean aphids (*Aphis fabae*) and a strain of the closely related species *Regiella insecticola* (U type) from *A. pisum* persisted *in vitro* when co-cultured with the C6/36 cell line from the mosquito *Aedes albopictus* or the S2 cell line from *Drosophila melanogaster*. The growth and abundance of *H. defensa* and *R. insecticola* in these cultures were not quantified but descriptions of their maintenance suggest both primarily persisted intracellularly at low abundance [40]. By contrast, *H. defensa* grows to high titres as *A. pisum* develop from first instar nymphs to adults [21,36].

Here, we report the *in vitro* culture of four *H. defensa* strains that exhibit variable protection against parasitoids *in vivo* owing to differences in APSE infections (table 1). We then demonstrate that one strain of APSE-infected *H. defensa* kills *A. ervi* in the absence of any aphid factor, and that horizontal transfer of this APSE to a non-infected strain of *H. defensa* confers the same killing activity.

2. Material and methods

(a) Insects and cell lines

Clonal *A. pisum* lines carrying the A2C, AS3, NY26 and ZA17 strains of *H. defensa* were maintained on fava bean plants at 20°C and a 16 L:8 D photoperiod as previously described [20,35–37]. *Aphidius ervi* used in the study were reared in the laboratory as a large population founded from commercially purchased (Arbico) and field-collected wasps on an *A. pisum* line with no facultative symbionts; adult wasps were provided continuous access to honey and water at 20°C under a 16 L:8 D photoperiod [36]. Culture media and insect cell lines used in the study are summarized in the electronic supplementary material, table S1.

Table 1. Characteristics of *H. defensa* strains cultured.

<i>H. defensa</i> strain	APSE variant	primary phage toxin	protection in aphids
A2C	no phage	no phage	none
NY26	APSE2	CdtB	moderate
AS3	APSE3	YDR	high
ZA17	APSE8	CdtB	moderate

(b) *Hamiltonella defensa* cultures

Acyrtosiphon pisum adults containing the A2C, AS3, NY26 or ZA17 strain of *H. defensa* were surface sterilized by submerging in 5% bleach, 1% Tween20 and 0.1% ROCCAL-D (Pfizer) (v/v in water) for 30 s followed by rinsing in sterile water for 30 s. Each aphid was then transferred to a 25 µl drop of medium and pierced using sterile forceps to release haemolymph from the body cavity into the medium. Each drop was then transferred to an individual culture well in a 24-well culture plate (Corning) containing 1 ml of medium with or without fetal bovine serum (FBS) and particular insect cell lines (electronic supplementary material, table S1). Primary cultures were maintained under ambient atmosphere at 20° or 27°C. Established cultures were passaged weekly or biweekly by adding 1×10^6 bacteria to 25 cm culture flasks (Falcon) containing 4.0 ml of TC100 medium plus 10% FBS with or without 1×10^6 TN5 cells (electronic supplementary material, table S1).

(c) Estimating *Hamiltonella defensa* titres

Cohorts ($n = 5$) of two-day old adult aphids infected with the A2C, AS3, NY26 or ZA17 strains of *H. defensa* were placed in 500 µl of phosphate buffered saline (PBS). The cuticle of each aphid was then pierced using forceps as described above. After removing the aphids, each drop was transferred to 1.5 ml micro-fuge tube and gently centrifuged at 50g to pellet aphid cells and debris. The supernatant was then transferred to a new tube and spun at 8000g for 10 min at 4°C to pellet *H. defensa*. After decanting the supernatant, DNA was extracted as described [21] and stored at –20°C. DNA was similarly isolated from *H. defensa* in established cultures by collecting 500 µl of medium 3, 6, 9 or 15 days after new cultures were passaged. *Hamiltonella defensa* titres were estimated by qPCR using specific primers for the single-copy *H. defensa dnaK* gene (electronic supplementary material, table S2) [21,35,47]. Ten microlitres of qPCR reactions contained 0.5 µl of 5 µM of each primer, 5 µl of the Rotor-Gene SYBR Green Master Mix and 1 µl of a 1:100 dilution of template and 3 µl of ultrapure water. Four technical replicates were run per sample using a Rotor-Gene Q cycler (Qiagen) with data acquired during the extension step. *In vivo* titre data were analysed by analysis of variance (ANOVA) while *in vitro* data were analysed by repeated-measures ANOVA.

(d) Diagnostic PCR

Diagnostic PCR reactions were run using specific primers that amplified unique domains in: (i) the *dnaK* gene present in all strains of *H. defensa*, (ii) the head protein gene (*p24*) present in all APSE variants, (iii) the *ydr* gene in APSE3 that persistently infects AS3, (iv) the *cdtB* gene in APSE2 and APSE8 that persistently infect NY26 and ZA17 respectively, (v) a hypothetical protein unique to ZA17, (vi) an *rtx-toxin* gene unique to NY26, and (vii) the *dnaK* gene of *B. aphidicola* (electronic supplementary material, table S2). PCR reactions (25 µl) contained 2.5 µl of 25 mM Mg²⁺, 0.5 µl of mixed dNTPs (2.5 µM each), 0.5 µl of

each primer (5 μ M), 1 μ l of a 1/100 dilution of template DNA, 0.2 μ l of HotMaster Taq DNA polymerase and 19.6 μ l of ultra-pure water. Samples were run using a T100 thermal cycler (Bio-Rad). Eight microlitres of each PCR reaction were then run on a 1% agar gel stained with ethidium bromide and visualized using a BioDoc-iT imaging system.

(e) Microscopy

Hamiltonella defensa were slide mounted and examined with a Leica DMXRXE upright epifluorescent microscope with differential interference optics and a Leica digital camera. Established cultures in flasks containing TN5 cells were also examined after staining with 20 μ g ml⁻¹ acridine orange (Sigma) or 1 μ g ml⁻¹ Hoechst 33342 (Sigma) in water using a Leica DM IRE2 inverted epifluorescent microscope with phase-contrast optics fitted with a Hamamatsu digital camera. *Aphidius ervi* eggs were stained with acridine orange and 1 μ g ml⁻¹ propidium iodide (Sigma) followed by examination as described above. Images were captured using Leica or SimplePCI software (Compix). For transmission electron microscopy (TEM), *H. defensa* and TN5 cells from established cultures were pelleted 4 days post-passage by centrifugation at 2000g at 4°C. After decanting the supernatant, the pellet consisting of bacteria and insect cells was fixed for 2 h in 0.1 M cacodylate-HCl buffer (pH 7.0) containing 2% glutaraldehyde (v/v), 2% paraformaldehyde (v/v) and 0.2% picric acid (v/v). After rinsing in 0.1 M cacodylate-HCl buffer and repelleting, cells were resuspended in 2% agar at 50°C followed by centrifugation for 10 min and storage at 4°C until the agar hardened. Agar-embedded samples were then post-fixed in 1% OsO₄ in 0.1 M cacodylate-HCl buffer for 1 h, rinsed in deionized water, and dehydrated in graded ethanols (30–100%) with 15 min wash steps. Samples were then rinsed 1× in acetone and 2× in propylene oxide for 15 min before infiltrating in propylene oxide: Epon-Araldite (1:1, 1:3) for 2 h. Samples were embedded in 100% Epon-Araldite, which polymerized over 24 h in a 75°C oven. Sections were cut with a Reichert Ultracut S ultramicrotome and stained with uranyl acetate and lead citrate. Sections were then examined and photographed using a JEOL JEM1011 (JEOL, Inc., Peabody, MA) TEM at 100 kV.

(f) *Aphidius ervi* assays

Acyrtosiphon pisum without facultative symbionts were individually parasitized by *A. ervi*. Aphids were surface sterilized as previously described 2 or 36 h post-parasitism and then dissected to isolate either newly laid *A. ervi* eggs or 1st instar larvae. One egg or larva was placed per well of a 96-well culture plate that contained 100 μ l TC100 + 30% FBS. A2C, AS3 or ZA17 from 3-day-old established cultures (greater than 50 generations old and capable of growing without TN5 cells) were then added to wells at a density of 1×10^4 to 1×10^6 bacteria ml⁻¹. *Aphidius ervi* eggs were allowed to develop for 72 h at 27°C while first instars were maintained for 48 h. Embryonic stages of *A. ervi* were recorded every 24 h with the treatment effects assessed by Fisher exact tests.

(g) APSE infectivity experiments

One millilitre of medium from 3-day old established cultures of the AS3 strain was collected and centrifuged at 8000g. Cell pellet template DNA was extracted as described above. The supernatant was collected and filtered through an 0.2 μ m syringe filter to eliminate all bacterial cells. Two microlitres of TURBO DNase (Ambion) and 5 μ l of buffer was then added to 50 μ l of this supernatant for 40 min at 37°C. This treatment degraded any naked DNA in the medium but cannot degrade DNA that is packaged inside of a virus particle. After adding EDTA (10 mM) to inactivate the DNase, supernatant template DNA was then extracted [21]. APSE-specific primers (electronic supplementary material, table S2)

were then used to amplify viral DNA using qPCR as above. APSE3 copy number ml⁻¹ of medium was calculated by multiplying the qPCR estimate by the dilution factor and elution volume. Two independently acquired biological replicates were performed for each treatment with samples internally replicated four times. A2C titre was determined by qPCR as described above. Six new A2C cultures were initiated by adding 1×10^7 bacteria to 4 ml of fresh TC100 + 10% FBS and 1×10^4 TN5 cells in a six-well culture plate (Corning). APSE3 in medium from the AS3 strain was then added to three A2C cultures ($n = 3$) at a multiplicity of infection (MOI) of 0.1 while three other A2C cultures were grown without APSE3. After 9 days at 27°C, 1 ml of medium was centrifuged followed by qPCR to determine APSE3 and *H. defensa* copy number in the cell pellet and APSE3 copy in the DNase pretreated supernatant. The effects of APSE3-infected A2C on development of *A. ervi* eggs were then compared with non-infected (phage-free) A2C by adding 1×10^6 bacteria per well and assessing the proportion of eggs that developed to the 128 cell stage as described above.

(h) Figure assembly and statistical analyses

Micrographs were assembled using ADOBE ILLUSTRATOR and PHOTOSHOP while figures were generated using R or GRAPHPAD PRISM 5.0. Statistical analyses were performed using R (www.r-project.org) or JMP PRO version 11 (SAS Institute Inc., Cary, NC).

3. Results

(a) *Hamiltonella defensa* grows extracellularly *in vivo* and *in vitro*

Previous qPCR estimates indicated that titres of APSE- and non-infected *H. defensa* increase in *A. pisum* from approximately 1×10^7 per first instar nymph to more than approximately 1×10^9 per 2-day old adult [21]. In the current study, we determined that the titres of A2C, AS3, NY26 and ZA17 specifically in the haemolymph of 2-day old adults exceeded 1×10^9 bacteria (electronic supplementary material, figure S1). Most *H. defensa* were also non-motile pleomorphic rods in plasma with no bacteria in haemocytes [48] (electronic supplementary material, figure S1). Collectively, these findings supported that most *H. defensa* persist and probably grow extracellularly in *A. pisum*. We, therefore, used adult haemolymph as the source of *H. defensa* for establishing primary cultures. We initially focused on the phage-free A2C strain and tested several different types of insect cell culture media with or without FBS and different insect cell lines (electronic supplementary material, table S1). At 27°C and ambient atmosphere we observed bacteria with identical morphology to *H. defensa* in all primary cultures including those containing C6/36 and S2 cells used by Darby *et al.* [40]. However, after 3 days bacteria only noticeably increased in abundance in culture wells containing TC100 medium plus 10% FBS and the TN5 cell line (electronic supplementary material, table S1). We, therefore, used these conditions to initiate primary cultures for the APSE-infected AS3, NY26 and ZA17 strains (electronic supplementary material, table S1). Similar to A2C, AS3 and ZA17 increased in abundance under ambient atmosphere at 27°C, whereas NY26 showed no increase in abundance at 27°C but did so when maintained at 18°C. Approximately 1×10^6 bacteria from primary cultures were transferred after 7 (A2C, AS3, ZA17) or 14 days (NY26) to 25 cm² cell culture flasks containing 4 ml of TC100 plus 10% FBS and 1×10^4

Figure 1. Growth of the A2C, AS3, NY26 and ZA17 strains and TN5 cells in established cultures. (a) Genome copy number (mean $\pm$ 95% confidence intervals) ml⁻¹ of medium for the A2C, AS3 and ZA17 strains over 9 days at 27°C as measured by qPCR. Each strain grew but not at the same rate as indicated by significant strain ($F = 212.8$; $p < 0.0001$) and day ($F = 1364.9$; $p < 0.0001$) effects (repeated-measures ANOVA). (b) Genome copy number ml⁻¹ of medium for the NY26 strain over 15 days at 18°C. Copy number significantly increased during this period ($F = 88.1$; $p < 0.0001$). (c) TN5 cells ml⁻¹ of medium over 9 days at 27°C when co-cultured with the A2C, AS3 and ZA17 strains or no *H. defensa*. Treatment ($F = 65.1$; $p < 0.0001$) and day ($F = 16.4$; $p < 0.0001$) are both significant (repeated-measures ANOVA) owing primarily to growth of TN5 cells in the no *H. defensa* treatment. (d) TN5 cells ml⁻¹ of medium over 15 days at 18°C when co-cultured with the NY26 strain or no *H. defensa*. Treatment ($F = 53.6$; $p < 0.0001$) and day ($F = 26.4$; $p < 0.0001$) are both significant (repeated-measures ANOVA) owing to growth of cells with no bacteria.

TN5 cells. Each strain was thereafter passaged weekly (A2C, AS3, ZA17) or biweekly (NY26) under the same conditions and henceforth called established cultures. Diagnostic PCR assays confirmed that each established culture contained only the strain of *H. defensa* that was present in the donor aphid (electronic supplementary material, figure S2).

(b) Established cultures grow as extracellular bacteria

In established cultures, each *H. defensa* strain increased from 1×10^6 to a maximum of approximately 1×10^{10} bacteria ml⁻¹ in 6 (A2C, AS3, ZA17) or 15 (NY26) days (figure 1a,b). TN5 cells co-cultured with each strain *H. defensa* exhibited little

increase in abundance relative to TN5 cells cultured without bacteria (figure 1c,d). However, almost no TN5 cells with and without bacteria were stained by propidium iodide, which indicated most were viable. TN5 cells co-cultured with each strain of *H. defensa* also remained attached to culture plates and morphologically looked identical to TN5 cells without bacteria. *Hamiltonella defensa* in established cultures were morphologically similar to bacteria in aphids, but commonly formed pairs or short chains of cells not observed in *A. pisum* (electronic supplementary material, figure S3a–d). Staining with the DNA-specific dye Hoechst 33342 also indicated that *H. defensa* in established cultures predominantly grew extracellularly (electronic supplementary material, figure S3e,f). This conclusion

was further supported by TEM analysis of the A2C, AS3 and ZA17 strains. Ultrastructure at low magnification showed an abundance of variably shaped *H. defensa* that were extracellular (electronic supplementary material, figure S4a,b). Higher magnification images further showed that the cell envelope of *H. defensa* consisted of an inner and outer membrane but lacked a symbiosome membrane [49] present in some intracellular symbionts including *Buchnera* (electronic supplementary material, figure S3c). Inspection of hundreds of TN5 cells identified only one that contained an *H. defensa* cell in the cytoplasm, which indicated bacteria rarely enter this cell type (electronic supplementary material, figure S3d). We stored established cultures of *H. defensa* by adding 15% glycerol (vol/vol) to culture medium. After thawing on ice, aliquots stored for more than 1 month grew similarly to continuously propagated cultures when re-inoculated into medium indicating all strains can be cryopreserved.

(c) Some strains of *Hamiltonella defensa* grow as pure cultures in liquid medium

After one year and more than 50 passages, we tested each strain for the ability to grow in TC100 plus 10% FBS but no TN5 cells. Results showed that the A2C, AS3 and ZA17 strains grew at similar rates and to similar densities (approx. $1 \times 10^{10} \text{ ml}^{-1}$) at 27°C as bacteria co-cultured with TN5 cells. Each strain could also be routinely passaged and maintained as a pure culture in TC100 plus 10% FBS but would not grow in TC100 in the absence of FBS. The NY26 strain in contrast could not be cultured in the absence of TN5 cells. Given these results, we reassessed whether primary cultures of A2C, AS3 and ZA17 could be established from adult aphids by adding haemolymph to TC100 plus 10% FBS and no TN5 cells. Repeated efforts failed to generate any primary cultures that grew. Attempts to propagate *H. defensa* from established cultures on TC100 plus 10% FBS agar plates under ambient or a microaerobic atmosphere also failed to produce colonies for any strain.

(d) AS3 disables development of *Aphidius ervi*

Similar to symbionts, most endoparasitoid wasps require specialized conditions for development that make *in vitro* culture difficult [50–52]. While no wasps in the genus *Aphidius* have been successfully reared to adulthood *in vitro*, prior studies established that *A. ervi* eggs develop to the first instar in TC100 plus 10% FBS [53]. *Aphidius ervi* eggs have a thin chorion (egg shell) and develop in the haemolymph of aphid hosts. Eggs undergo complete cleavage to initially form a 128 cell embryo that is enveloped by an extraembryonic membrane of polar body origin within the chorion [53]. The embryo then ruptures from the chorion as a spherical morula that completes development into a first instar wasp larva [53]. Previous *in vivo* assays further showed that *A. ervi* eggs: (i) usually die during embryogenesis in *A. pisum* containing APSE3-infected *H. defensa*, (ii) exhibit lower levels of mortality that can occur before or after completion of embryogenesis in *A. pisum* containing APSE2- or APSE8-infected *H. defensa*, and (iii) exhibit no mortality in *A. pisum* containing phage-free *H. defensa* [36]. We thus assessed whether established cultures of *H. defensa* affected development of *A. ervi* eggs by focusing on the APSE3-infected AS3, APSE8-infected ZA17 and phage-free A2C strains. We did not bioassay APSE2-infected NY26 because of its low-temperature

requirements and APSE2 contains a similar toxin gene cassette, including *cdtB*, as APSE8 [36].

At a starting density of 1×10^6 bacteria ml^{-1} , AS3 significantly reduced the proportion of newly laid eggs that developed into 128 cell stage embryos, whereas A2C and ZA17 did not (figure 2a). AS3 at $1 \times 10^5 \text{ ml}^{-1}$ also significantly reduced the proportion of *A. ervi* eggs that developed to the 128 cell stage but 1×10^4 bacteria ml^{-1} did not (figure 2a). Staining with propidium iodide showed that eggs co-cultured with AS3 initially exhibited reduced viability of cells forming the extraembryonic membrane at 24 h post-culture (figure 2b). This was followed by mortality of most cells in the extraembryonic membrane plus many embryonic cells by 48 h (figure 2b). By contrast, most embryos showed no loss of viability at 24 or 48 h when co-cultured with ZA17, A2C or no *H. defensa* (figure 2b). We next cultured AS3 or A2C (negative control) at a starting density of $1 \times 10^6 \text{ ml}^{-1}$ for 6 h in TC100 plus 10% FBS. We then removed the bacteria and added newly laid *A. ervi* eggs to the conditioned medium. Most *A. ervi* eggs in AS3-conditioned medium failed to develop to the 128 cell stage, while most eggs in A2C-conditioned or non-conditioned (control) medium developed to the 128 cell stage (figure 2c). To assess whether ZA17 affected *A. ervi* larvae, bacteria were co-cultured with first instars followed by assessment of larval viability after 24 and 48 h. Results revealed no reduction in viability when compared with larvae co-cultured with A2C or no bacteria (figure 2d).

(e) APSE3 confers anti-*Aphidius ervi* defence to the A2C strain

Comparative genomic data indicate that all APSEs persist as integrated proviruses in *H. defensa* genomes, while other results indicate that some APSE variants replicate [19,21,28]. Phylogenetic evidence also suggests that APSEs have horizontally moved among *H. defensa* strains [25]. However, no experimental evidence supports that any APSE is infectious or that phage acquisition alters the phenotype of strains in defence against parasitoids. As APSE3-infected AS3 disabled development of *A. ervi*, we assessed whether established cultures produced APSE3 that could infect phage-free A2C. First, we measured the abundance of bacteria and APSE3 in a 5-day old culture of AS3 by centrifuging 1 ml of medium to produce a bacterial pellet and supernatant. qPCR assays using DNA isolated from the pellet showed that copy number of APSE3 and AS3 were similar, which supported that approximately 1 copy of the APSE3 genome was present per bacterial cell (figure 3a). However, assays also showed that DNase pretreated supernatants contained 2.7×10^8 copies of the APSE3 genome, which supported that established cultures of AS3 produced APSE3 particles (figure 3a). We, therefore, used this supernatant to inoculate cultures containing 1×10^7 A2C at an MOI of 0.1 to assess whether APSE3 could infect a phage-free strain of *H. defensa*. qPCR analysis 9 days post-infection detected a 10-fold higher copy number of the APSE3 versus A2C genome in DNA templates from pelleted bacterial cells, and 4×10^{10} copies of the APSE3 genome ml^{-1} in DNase pre-treated supernatants (figure 3b). This outcome strongly supported that APSE3 infected A2C, which also resulted in production of APSE3 particles. However, it also indicated that infection was not fully fatal to A2C as large numbers of bacteria still survived. We, therefore, compared the effects of APSE3-infected A2C to non-infected A2C on development of *A. ervi* by adding 1×10^6 bacteria to culture wells containing 1 ml of

Figure 2. The AS3 strain of *H. defensa* disrupts development of *A. ervi* during early embryogenesis. (a) Proportion of *A. ervi* eggs that developed to the 128 cell stage after 36 h when co-cultured with the AS3, A2C or ZA17 strains with no *H. defensa* (Control). Starting densities ml^{-1} are indicated on the x-axis. Asterisks (*) indicate treatments that significantly differed from the no bacteria control ($\chi^2_5 = 62.7$, $p < 0.0001$ followed by post hoc pairwise Fisher exact tests). (b) Epifluorescent micrographs of *A. ervi* eggs co-cultured with AS3, A2C, ZA17 or no bacteria for 24 or 48 h followed by staining with propidium iodide (red) and acridine orange (green). Several cells in the extraembryonic membrane of eggs co-cultured with AS3 are stained by propidium iodide at 24 h (arrowheads) while many embryonic cells are stained at 48 h. By contrast, eggs cultured with A2C, ZA17 or no *H. defensa* are only stained by acridine orange indicating no loss of viability. Eggs cultured with A2C, ZA17 or no bacteria exhibit little or no staining by propidium iodide. The morula (arrowhead) that ruptured out of the chorion (arrow) is visible at 48 h for the image shown under A2C, while only a morula is shown at 48 h under ZA17 and no *H. defensa*. Scale bar equals 200 μm . (c) Asterisk (*) indicates that the proportion of *A. ervi* eggs that developed to the 128 cell stage at 36 h was significantly lower in medium conditioned by AS3 than medium conditioned by A2C or medium with no *H. defensa* ($\chi^2_2 = 61.2$, $p \leq 0.0001$ followed by post hoc pairwise Fisher exact tests). (d) Proportion of viable first instar *A. ervi* after 48 h did not differ (n.s.) when cultured with ZA17 or no *H. defensa* ($\chi^2_1 = 0$, $p = 1$).

Figure 3. AS3 produces APSE3 that infects A2C and alters its ability to kill *A. ervi* embryos. (a) Genome copy number (mean $\pm$ 95% confidence intervals) of AS3 and APSE3 in 1 ml of an established 5-day old culture. Data generated from analysis of two independent cultures after centrifuging medium and isolating DNA from the bacterial pellet and DNase pretreated supernatant. (b) Genome copy of A2C and APSE3 in 1 ml of an A2C culture 9 days post-infection with virus from an AS3 culture. Data generated from analysis of three independent cultures after centrifuging medium and isolating DNA from the bacterial pellet and DNase pretreated supernatant. The left side of the graph shows mean copy number for A2C and APSE3 in the bacterial pellet, and APSE3 in medium pretreated with DNase. The right side of the graph shows genome copy number for A2C in 1 ml of medium from 9-day old cultures that were not infected with APSE3. (c) Proportion of *A. ervi* eggs that developed to the 128 cell stage after 36 h when co-cultured with A2C that were infected 9 days earlier by APSE3 or that were uninfected. Both treatments were bioassayed by adding 1×10^6 bacteria to 1 ml of medium containing newly laid *A. ervi* eggs. The asterisk (*) above the bar for APSE3-infected A2C indicates this treatment significantly differed from the uninfected A2C control ($\chi^2_1 = 66.5$, $p < 0.0001$).

medium and newly laid wasp eggs. Results showed that APSE3-infected A2C reduced the proportion of eggs that developed to the 128 cell stage relative to uninfected A2C (figure 3c). This reduction was also nearly identical to the effects of APSE3-infected AS3 (figure 3a).

4. Discussion

No obligate and only a few facultative heritable symbionts of insects have been cultured outside of hosts. One difficulty in culturing these organisms is that heritable symbionts are generally restricted to host tissues and genome reduction results in many species having specialized requirements for growth that are difficult to mimic *in vitro* [43]. Another challenge is that the slow growth of many heritable symbionts elevates the risk of contamination when trying to establish primary cultures [43]. Most successes in culturing heritable insect symbionts have used insect cells that could serve as hosts for intracellular growth along with media developed for culture of insect cells rather than bacteria [40–42,44].

Given these precedents, we took a similar approach to culturing *H. defensa*. However, our priorities focused on identifying conditions in which strains of *H. defensa* that vary in levels of *in vivo* protection against *A. ervi* grow to comparable titres as in the aphid *A. pisum*. This allowed us to conduct experiments *in vitro* that would indicate whether symbiont products from different strains disable development of *A. ervi*. Consistent with *in vivo* results [20], *in vitro* assays indicate that APSE3-infected AS3 disable embryonic development of *A. ervi*. However, our *in vitro* data differ from prior *in vivo* studies

by, to our knowledge showing for the first time that: (i) AS3 kills *A. ervi* eggs in the absence of any aphid-produced product, and (ii) mortality is owing to a soluble factor released from *H. defensa*/APSE that can enter wasp eggs prior to rupturing from the chorion as a morula. We also found that while cytotoxic to *A. ervi* this factor does not reduce the viability of TN5 cells, although TN5 cell growth was lower when co-cultured with *H. defensa* when compared to growth without bacteria. This finding is consistent with observations that wasps are killed *in vivo*, but aphids suffer relatively minor costs to infection with *H. defensa* [31].

Also consistent with prior *in vivo* studies [20], we found that the phage-free strain A2C did not affect *A. ervi* development. However, our results show that APSE3 from AS3 infects A2C, which then gains the ability to disable *A. ervi* development. This indicates that some APSE variants retain the capacity to infect *H. defensa* and horizontally transfer the factor(s) that kill *A. ervi* embryos. While host restriction inevitably leads to heritable symbiont decay over time [54], phage-mediated horizontal transfer of ecologically important traits between heritable symbionts provides a mechanism to maintain symbiont innovation. By contrast, our finding that ZA17 infected by APSE8 did not affect development of *A. ervi* eggs or larvae *in vitro* suggests interactions with aphid factors are required for disabling wasp development *in vivo*. Consistent with this, *H. defensa* infected with APSE8 confer little protection against *A. ervi* in some aphid genotypes but moderate protection in others [29].

Our results identified TC100 plus 10% FBS as the preferred medium for *H. defensa* growth. However, we could not establish primary cultures from aphids in this medium without

TN5 cells, although the A2C, AS3 and ZA17 strains could be propagated as pure liquid cultures after multiple passages *in vitro*. We observed lower survival and/or growth of *H. defensa* in the other media and cell lines we tested including C6/36 and S2 cells. Thus, culture conditions probably account for the slow *in vitro* growth and intracellular persistence reported previously for *H. defensa* [40]. No cell lines have been established from aphids but our results are consistent with other studies showing that cells from non-host insect species benefit the culture of some heritable symbionts [40–44]. The near absence of intracellular *H. defensa* in TN5 cells strongly argues that infection of this cell line is not essential for persistence or growth of *H. defensa*. What TN5 cells provide that is required for initiating primary cultures and maintenance of the NY26 strain remains unclear. Unknown adaptations by A2C, AS3 and ZA17 after multiple passages *in vitro* could play a role in why these strains could be shifted to growth in pure liquid cultures. However, this shift did not alter the ability of the AS3 strain with APSE3 to kill *A. ervi* eggs or for APSE3 transfer to confer *A. ervi*-killing activity to the A2C strain. The factors that underlie the requirement of NY26 for culture at lower temperatures with TN5 cells will also require further study.

In summary, our results provide an essential foundation for identifying the *H. defensa*-associated molecules that provide protection to aphids against parasitoids. They also provide

new opportunities for understanding APSE-*H. defensa* interactions, which are of interest given our results show that APSE3 replicates but does not cause high level mortality of *H. defensa*. The ability to propagate particular strains of *H. defensa* in pure cultures also provides opportunities for characterizing genomic and transcriptome strain variation and potentially manipulating this symbiont genetically.

Ethics. The authors declare that this research (i) has not been published previously elsewhere, (ii) was not misconducted, (iii) did not involve animal treatment, and (iv) did not involve plagiarism in any form.

Data accessibility. *In vitro* culture media tested, PCR primers used in the study, diagnostic PCR results and microscopy images are provided as the electronic supplementary material. Datasets used to generate figures 1a–d, 2a,c,d and 3a–c are also provided as electronic supplementary material.

Authors' contributions. M.R.S. and K.M.O. conceived the study; M.R.S., J.W.B., G.C. and K.M.O. designed the study; J.W.B., G.C. and M.R.S. collected data; J.W.B., G.C. and M.R.S. analysed data. M.R.S. drafted the manuscript with J.W.B., G.C. and K.M.O. contributing to writing of the paper.

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