Amino acid transporters implicated in endocytosis of *Buchnera* during symbiont transmission in the pea aphid

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**Abstract**

**Background:** Many insects host their obligate, maternally transmitted symbiotic bacteria in specialized cells called bacteriocytes. One of the best-studied insect nutritional endosymbioses is that of the aphid and its endosymbiont, *Buchnera aphidicola*. Aphids and *Buchnera* are metabolically and developmentally integrated, but the molecular mechanisms underlying *Buchnera* transmission and coordination with aphid development remain largely unknown. Previous work using electron microscopy to study aphid asexual embryogenesis has revealed that *Buchnera* transmission involves exocytosis from a maternal bacteriocyte followed by endocytotic uptake by a blastula. While the importance of exo- and endocytic cellular processes for symbiont transmission is clear, the molecular mechanisms that regulate these processes are not known. Here, we shed light on the molecular mechanisms that regulate *Buchnera* transmission and developmental integration.

**Results:** We present the developmental atlas of ACYPI000536 and ACYPI008904 mRNAs during asexual embryogenesis in the pea aphid, *Acyrthosiphon pisum*. Immediately before *Buchnera* invasion, transcripts of both genes were detected by whole-mount in situ hybridization in the posterior syncytial nuclei of late blastula embryos. Following *Buchnera* invasion, expression of both genes was identified in the region occupied by *Buchnera* throughout embryogenesis. Notably during *Buchnera* migration, expression of both genes was not concomitant with the entirety of the bacterial mass but rather expression colocalized with *Buchnera* in the anterior region of the bacterial mass. In addition, we found that ACYPI000536 was expressed in nuclei at the leading edge of the bacterial mass, joining the bacterial mass in subsequent developmental stages. Finally, quantitative reverse transcription real-time PCR suggested that early in development both transcripts were maternally provisioned to embryos.

**Conclusions:** We venture that ACYPI000536 and ACYPI008904 function as nutrient sensors at the site of symbiont invasion to facilitate TOR-pathway-mediated endocytosis of *Buchnera* by the aphid blastula. Our data support earlier reports of bacteriocyte determination involving a two-step recruitment process but suggest that the second wave of recruitment occurs earlier than previously described. Finally, our work highlights that bacteriocyte-enriched amino acid transporter paralogs have additionally been retained to play novel developmental roles in both symbiont recruitment and bacteriome development.

**Keywords:** Amino acid transporter, Aphid development, Coevolution, Host/symbiont developmental integration, Symbiosis

**Background**

A compelling challenge in symbiosis research concerns identifying and characterizing the mechanisms of host/symbiont metabolic and developmental integration [1]. Recently, we began to address this challenge by studying host/symbiont developmental integration in the pea aphid *Acyrthosiphon pisum* and its obligate endosymbiont *Buchnera aphidicola* [2]. Like all aphids, *A. pisum* feeds exclusively on plant phloem sap, a diet that is rich...
in sugar but limited in provision of essential amino acids and vitamins [3–5]. To overcome the nutritional deficiency of their diet, aphids anciently acquired B. aphidicola, an endosymbiont that metabolically compensates the nutritional shortcomings of their diet [1, 6, 7]. Buchnera is transmitted from generation to generation through transovarial transmission [8], and remarkably aphids cured of Buchnera infection fail to reproduce [9–12].

Aphids are cyclical parthenogens, capable of both sexual and asexual modes of reproduction. In asexual aphids, one ovariole contains a series of embryos at various developmental stages [13]. Parthenogenetic embryogenesis has been described by Will [14] and Miura et al. [8]. The parthenogenetic embryo originates from a maturation division of the oocyte by skipping the reduction division of meiosis I, producing a polar body and an oocyte nucleus [8, 15]. The oocyte nucleus is considered to be the incipient parthenogenetic embryo that divides synchronously five times in a common cytoplasm to form the syncytial embryo with 32 syncytial nuclei. Cellularization of the syncytial nuclei then generates the blastula embryo. Primordial germ cells that are specified in the posterior region of the blastula separate the remaining uncellularized syncytial nuclei into two locations: (1) the central syncytium (in the center of the blastoderm) and (2) the posterior syncytium (at the posterior end of the blastoderm) [8].

Developmental integration of Buchnera into host aphids starts with transmission of a small population of Buchnera from maternal bacteriocytes, the specialized aphid cells accommodating Buchnera, to a blastula [8]. Transmission of Buchnera involves exocytosis from maternal bacteriocytes followed by endocytosis by the developing blastula. During exocytosis, individual Buchnera lose their host-derived symbiosomal membrane (a perisymbiont membrane that encloses individual Buchnera cells) and migrate naked across the extracellular space between the maternal bacteriocyte and the posterior pole of the blastula to reacquire their symbiosomal membrane by endocytotic passage into the blastoderm (stage 7 of development) [8, 16]. Following transmission, the Buchnera population, together with aphid uncellularized syncytial nuclei, are subdivided into uninucleate bacteriocyte cells [8]. After this, bacteriocyte cells proliferate [17], and maturation of the bacteriome proceeds [2]. Aphid bacteriomes, the organ of symbiosis, comprise two cell types: bacteriocytes that house Buchnera and intercalating sheath cells that do not house Buchnera [18, 19]. Localization of the transcription factor Distal-less (Dll) reveals that bacteriocytes are recruited through two steps. In the first step, bacteriocyte cells originate from the syncytial nuclei that localize prior to Buchnera invasion in the posterior syncytium of the blastoderm embryo [17]. In the second step, bacteriocyte cells originate from approximately 40–60 Dll-expressing cells that migrate across the germband and intercalate between the original bacteriocytes during germband extension [17]. In contrast to the original proposal by Braendle et al. [17] that bacteriocytes are recruited in two steps, Koga et al. [16] hypothesized, on the basis of the smaller nuclei of the second group of cells and their localization between the original bacteriocyte population, that the second group of cells may in fact be sheath cells and not bacteriocytes. In late embryogenesis, aphid embryos undergo katatresis when the head is repositioned from the posterior to the anterior pole of the egg chamber resulting in inversion of the body axis; during this maturation, bacteriocyte cells are positioned to the dorsal side of the embryo where they later form the mature bacteriome [2, 8, 16].

Work to date in asexually reproducing A. pisum has demonstrated that specification of bacteriocytes occurs independently of symbiont invasion from mother to progeny [17] and that Buchnera transmission involves exo- and endocytotic cellular processes [16]. Bacteriocytes are widely found in sap-feeding and blood-feeding insect lineages, and yet little is known about the molecular network underlying bacteriocyte morphogenesis or the mechanisms that regulate transmission and developmental integration of symbionts.

Here, by studying the expression of two aphid amino acid transporter mRNAs during asexual embryogenesis, we gain insight into the molecular mechanisms that regulate transmission and developmental integration of Buchnera into aphid hosts. Amino acid transporters are the membrane proteins responsible for cellular nutrient exchange and nutrient sensing [20]. As nutrient sensors, amino acid transporters act as transceptors: dual-function molecules that both transport amino acids and function as receptors sensing extracellular amino acid availability and modulating intracellular nutrient-responsive signaling pathways [20–23]. Amino acid transporters have been implicated in a broad diversity of cellular and developmental functions that include control of cell growth [24–27], cell proliferation [28–30], regulation of translational capacity [31], lysosome biogenesis [32], and coordination of endocytosis/phagocytosis [26, 33]. The A. pisum genome encodes 40 putative amino acid polypeptide organocation superfamilies [34]. So far, bacteriocyte-enriched amino acid transporters have been implicated in regulating host and symbiont-mediated amino acid biosynthesis in bacteriocytes [35], and comparative work has demonstrated that retention of duplicated amino acid transporters can result from
selection to function in symbiosis [36–38]. Within A. pisum bacteriocytes, the two amino acid transporters that are most highly expressed are the eukaryotic-specific amino acid/auxin permease (AAAP) family (TC# 2.A.18) member, ACYPI000536, and the amino acid/polypeptide transporters family (TC# 2.A.3) member, ACYPI008904, and ACYPI008904 are gene identifiers that have been assigned in the ACFyrtosiphon Pism reference genome available at AphidBase [39]. To enhance readability in this paper, we will refer to ACYPI000536 as AAAP-536 and ACYPI008904 as APC-8904. Using whole-mount in situ hybridization and quantitative PCR, we present the developmental atlas of AAAP-536 and APC-8904 mRNAs during asexual embryogenesis. In interpreting the developmental atlas of AAAP-536 and APC-8904 expression, we propose that these amino acid transporters play novel roles in endocytosis of Buchnera by aphid blastula and in bacteriocyte development during asexual aphid embryogenesis.

Methods

Aphids

Acyrtosiphon pisum line LSR1 [40] and a Taiwanese line NTU [41] were maintained on Vicia faba and incubated at 20 °C under a 16-h light/8-h dark cycle. Oocytes and embryos for whole-mount in situ hybridization were dissected from wingless adult females in phosphate-buffered saline (PBS; 10 mM phosphate buffer, 154 mM NaCl, pH 7.4; Sigma-Aldrich). Oogenesis and embryogenesis staging was according to Miura et al. [8] and germ cell locations according to Chang et al. [42].

Cloning of AAAP-536 and APC-8904 gene fragments

Primer pairs for amplifying AAAP-536 and APC-8904 gene fragments were designed via MacVector version 7.2.2 (Accelrys Inc.) according to the gene sequences in AphidBase 2.1 [39]. Primers designed to AAAP-536 coding sequence were further checked using Primer-BLAST against the pea aphid genome Acyr_2.0 assembly, confirming that each primer hit only its isoform targets (amplicon located at 1719–2387 of XM_008185850.2 and 1150–1818 of NM_001246304.1). Due to the high similarity of APC-8904 nucleotide sequence to that of its paralogs: 82.56% coding sequence similarity to ACYPI008323 and 76.43% coding sequence similarity to ACYPI002633, we designed APC-8904 gene-specific primers for hybridization to the 3′ untranslated region (UTR) of APC-8904. Nucleotide similarity in the 3′ UTR of APC-8904 and its paralogs is much lower such that similarity between APC-8904 and ACYPI008323 is 66.32% across the 193 base pairs (bp) of alignable 3′ UTR sequence and that between APC-8904 and ACYPI002633 is 37.99% across the 766 bp of alignable 3′ UTR sequence. For AAAP-536, 669 bp of open reading frame (ORF) was amplified from a plasmid containing the gene’s full-length cDNA [35] with forward primer 5′-TGGGATGCTAAGTTTACCTCC-3′ and reverse primer 5′-TTCAATGGTAGAAATTATAGTGTT-3′. For APC-8904, 566 bp of the 3′UTR was amplified from whole insect LSR1 cDNA [35] with forward primer 5′-TTCTCCCTATGGTTCCACACACCGTC-3′ and reverse primer 5′-TTCTCAAGCGGAACACACCGTCT-3′. Amplification of gene fragments was performed starting with an initial denaturation at 94 °C for 30 s, followed by 30 cycles at 94 °C for 10 s, 50 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. Both amplicons were subcloned into pGEM-T vector (Promega), and the insert sequences and orientations were confirmed by DNA sequencing before RNA probe synthesis.

Whole-mount in situ hybridization and microscopy

Hybridizations in situ with digoxigenin (DIG)-labeled RNA probes to whole-mount ovaries were performed as described by Chang et al. [43]. The antisense and sense DIG-labeled RNA probes were transcribed with SP6 or T7 RNA polymerase (New England Biolabs). Transcription was carried out from 1 μg linearized plasmid DNA in 20 μl reaction mixtures containing 2 μl 10 × RNA polymerase reaction buffer (400 mM Tris–HCl, 60 mM MgCl₂, 20 mM spermidine, 10 mM dithiothreitol, ρH 7.9; New England Biolab), 2 μl 10 × DIG RNA labeling mix (Roche), 0.5 μl RNase inhibitor (Invitrogen), and 2 μl RNA polymerase (New England Biolabs). The reaction mixtures were incubated for 4 h at 37 °C, and the products were verified by size on a 1.5% agarose gel containing 2.2 M formaldehyde. After adding sodium acetate to 0.3 M, nucleic acids were precipitated with ethanol until further use.

Dissected ovaries from A. pisum line LSR1 containing developing oocytes and embryos were fixed in 3.8% formaldehyde (VWR) in PBS at 4 °C overnight. Each experiment included antisense riboprobe (experimental group) and sense riboprobe (control group) treatments, and the working concentration for each riboprobe was 2.0 ng/μl. Other steps followed the protocol of Chang et al. [43]. Probe hybridizations for AAAP-536 and APC-8904 were carried out at 68 and 60 °C, respectively, and specimens were incubated overnight at 4 °C with anti-DIG Fab fragments conjugated to alkaline phosphatase (Roche), diluted 1:500 in 1× blocking reagent (Roche). Nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche) was applied to detect the hybridized probes. We terminated the substrate reaction concurrently for experimental (antisense riboprobe) and control (sense riboprobe) samples based on the relative intensity of signal in the region of embryos occupied by...
and sense riboprobe (control) treatments. Following signal development, samples were counterstained with 2 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at room temperature for 1 h and mounted in 70% glycerol (Sigma-Aldrich) in PBS at 4 °C overnight. Photographs of NBT/BCIP staining were acquired with a Zeiss Axiovert 200 microscope connected to a Zeiss AxioCam ICc1 camera. Photographs of DAPI staining were taken with the Leica SP5 laser scanning confocal microscope in the University of Miami, Department of Biology Microscopy Core Facility. The experiment was performed 13 times for AAAP-536 and 6 times for APC-8904. Samples collected from each developmental stage were counted to report on the repeatability of our results; count data are available in Additional file 1: Table S1 for

**Real-time PCR**

Total RNA for each biological replicate was prepared from asexual adult female ovarioles (equivalent to 600–700 ovarioles) from *A. pisum* line NTU. Each ovariole contains germaria, oocytes and developing embryos at various stages of development enveloped within egg chambers. Dissection was performed under a Leica EZ4 Stereo Microscope with non-removable 16× eyepieces at 40× magnification on a 12-well watch glass with one individual adult dissected into each well. To avoid contamination, ovaries were separated from other somatic tissues and the somatic tissues were removed using a 200-μl pipetman (Eppendorf). Ovaries were washed several times with ice-cold PBS until no maternal bacteriocytotes or other somatic tissue remained in the well. Washes were performed with great care to avoid damaging the ovary structure. Under 56× magnification on the Leica Stereo Microscope, using a black enameled pin (diameter 0.25 mm; Entomoravia), the egg chambers were separated on the follicular stalk between embryos that were 0.25 mm; Entomoravia), the egg chambers were separated from embryos before stage 5 using 2× NBT/BCIP staining were acquired with a Zeiss Axiovert 200 microscope connected to a Zeiss AxioCam ICc1 camera. Photographs of DAPI staining were taken with the Leica SP5 laser scanning confocal microscope in the University of Miami, Department of Biology Microscopy Core Facility. The experiment was performed 13 times for AAAP-536 and 6 times for APC-8904. Samples collected from each developmental stage were counted to report on the repeatability of our results; count data are available in Additional file 1: Table S1 for AAAP-536 and Additional file 2: Table S2 for APC-8904.

**Cytological staining**

Dissected ovaries from *A. pisum* line NTU containing developing oocytes and embryos were fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS at room temperature for 20 min. Following fixation, ovaries were washed with 0.2% of Triton-X 100 (Sigma-Aldrich) in PBS three times, 10 min for each wash. Ovaries were then incubated with 2 μg/ml of DAPI (Sigma-Aldrich) at room temperature for 1 h and mounted in 70% glycerol (Sigma-Aldrich) in PBS at 4 °C overnight. Photographs of DAPI staining were taken with the Leica SP5 laser scanning confocal microscope at National Taiwan University.
Results
The developmental atlas of AAAP-536 and APC-8904 expression with reference to the symbiotic bacterial mass
We detected the expression of AAAP-536 and APC-8904 mRNAs in oocytes and early embryos of asexual A. pisum using whole-mount in situ hybridization with DIG-labeled RNA probes. AAAP-536 expression was not detected in the newly segregated oocyte (Fig. 1a), mature divided oocyte (Fig. 1b), syncytial embryos (Fig. 1c, d), or early blastoderm embryos (Fig. 1e). Expression of AAAP-536 was first identified in the nuclei within the posterior syncytium of the stage 6 blastoderm embryos (Fig. 1f; Additional file 4: Figure S1). After stage 6, a maternal symbiotic bacterial mass invaded the embryos (developmental stage 7) from the posterior opening between the enlarged follicle cells. Strong expression of AAAP-536 was identified in the region of the bacteria during the time that the bacterial mass was mixing with the posterior syncytial nuclei (Fig. 2a). To understand the relationship between the localization of syncytial nuclei and the bacterial mass, refer to Additional file 5: Figure S2 and note that panel g presents the same developmental stage as presented in Fig. 2a). Once most of the symbiotic bacterial mass has flowed to the embryo, gastrulation is initiated and germ band invagination begins (developmental stages 8 and 9). At this point, we observed preferential expression of AAAP-536 in the anterior region of the bacterial mass (where the posterior syncytial nuclei were located), and progressively weaker expression of AAAP-536 in the posterior tail-like region of the bacterial mass (Fig. 2b, c, indicated with arrows). Unexpectedly, some transcripts of AAAP-536 were detected in the periphery of nuclei outside of the bacterial mass after invasion of endosymbionts at developmental stages 8 and 9 (Fig. 2b, c, indicated with arrowheads). We infer that these AAAP-536-positive nuclei are the syncytial nuclei of the central syncytium and that later they are incorporated with the bacterial mass as bacteriocyte nuclei. AAAP-536 signals remained in the bacterial region of the gastrula, from stage 8 to stage 10 (Fig. 2b–d). Later in development, at...
the time that the germband undergoes segmentation and limb bud formation, the bacterial mass compartmentalizes, with individual bacteriocyte cells becoming conspicuous (stages 10–14). At this time, transcripts of *AAAP-536* remained associated with the symbiotic bacterial mass (stages 11–14, Fig. 3a–d) and relatively strong signals of *AAAP-536* were found around the nuclei of bacteriocytes in segmented embryos at stage 11 and stage 12 (Fig. 3a, b, indicated with arrowheads). Strong expression of *AAAP-536* was also detected in embryonic bacteriocytes during limb bud formation (stages 13–14) (Fig. 3c, d). *AAAP-536* expression in bacteriocytes continued following completion of segmentation (stage 14) through katatrepsis (stage 15) (Fig. 3e). Following katatrepsis, bacteriocytes are grouped into the bilobed structure called the bacteriome. While *AAAP-536* expression was found primarily in the embryonic bacteriome (Fig. 4a–d), *AAAP-536* signals were additionally observed in the follicular epithelium at the egg chamber surface from stage 13 (Figs. 3c–e, 4b–d, indicated with arrows) and as non-specific signal in the terminal region of the rostrum in embryos after stage 18 (Fig. 4e, indicated with yellow arrowhead).

Unlike *AAAP-536*, we observed ubiquitous background signals of *APC-8904* in the gerarium, the newly segregated oocyte (Fig. 1g), and mature divided oocyte
In syncytial embryos and early blastoderm embryos, transcripts of *APC*-8904 were randomly distributed (Fig. 1i–k, indicated with arrowheads). Similar to *AAAP*-536, in late stage 6 blastoderm embryos, we observed preferential localization of *APC*-8904 transcripts in the posterior syncytium (Fig. 1l); importantly, these preferential posterior syncytium *APC*-8904 signals were not found in the sense control group (Fig. 1m, n). Similar to *AAAP*-536, we observed transcripts of *APC*-8904 in the region occupied by the bacterial mass: during *Buchnera* invasion (Fig. 2e–g), bacteriocyte formation (stages 10–14) (Figs. 2h, 3f–i), and bacteriome maturation (stages 15–20) (Figs. 3j, 4f–i). To summarize, we highlight the similarities and differences in expression of *AAAP*-536 and *APC*-8904 mRNAs. With respect to similarities, (1) preferential expression of both transporter genes was first recognized in nuclei of the posterior syncytium of the blastula (developmental stage 6 in Fig. 1f, l); (2) transcripts of both genes did not always colocalize with all *Buchnera* cells (Fig. 2b, c, f, g, h, indicated with arrows), but rather both appeared at the *Buchnera* migration front such that we observed colocalization of both transcripts and *Buchnera* in the anterior region of the bacterial mass (stages 8–9); and (3) signals for both transporters were detected during *Buchnera* invasion, bacteriocyte formation, and bacteriome maturation (Figs. 2, 3, 4). In contrast, where *AAAP*-536 transcripts were detected in the periphery of nuclei outside the bacterial region during *Buchnera* invasion (Fig. 2b, c, indicated with arrowheads), transcripts of *APC*-8904 were not (Fig. 2f, g).

**AAAP-536 and APC-8904 were expressed very early in development**

We were intrigued by the expression of *AAAP*-536 and *APC*-8904 immediately prior to *Buchnera* invasion in the posterior syncytium of stage 6 embryos and wondered whether transcript detection so early in development was the result of maternal provisioning or zygotic expression. Thus, we further interrogated the start point of *AAAP*-536 and *APC*-8904 gene expression patterns in two ways. First, we reduced the stringency of in situ
hybridization conditions for testing the earliest expression of AAAP-536—before stage 6—by decreasing the hybridization temperature to 60 °C in the hope that we would increase our ability to detect early signal if the control group remained clean under the reduced stringency hybridization condition. Unfortunately, reducing the stringency of the hybridization conditions resulted in detection of ubiquitous signals in the sense control group making it impossible to obtain more information regarding the expression of AAAP-536 before stage 6 by in situ hybridization (Additional file 6: Figure S3). Given that relaxing the stringency of hybridization conditions did not facilitate obtaining greater insight, we then utilized quantitative reverse transcription real-time PCR, an approach that is more sensitive than in situ hybridization, to detect AAAP-536 and APC-8904 transcripts before stage 5 and after stage 6 of embryogenesis. While AAAP-536 and APC-8904 expression was high after stage 6, we found no significant difference in AAAP-536 and APC-8904 expression in embryos before stage 5 and after stage 6 (Fig. 5). This result was also repeated using a genetically discrete line of A. pisum, LSR1 (Additional file 7: Figure S4). Our results in both A. pisum lines demonstrated that AAAP-536 and APC-8904 were transcribed before stage 5 (Fig. 5 and Additional file 7: Fig. S4). Previously it has been argued that transcripts detected before stage 6 of development are of maternal origin, while those detected after stage 6 originate from the zygote [52, 53]. If this is the case, we infer that AAAP-536 and APC-8904 were maternally provisioned during early asexual aphid embryogenesis in A. pisum.

**Discussion**

**Developmental integration of Buchnera into aphid hosts**

Aphid work that targets elucidating the mechanisms of host/endosymbiont developmental integration has shown that: (1) specification of bacteriocytes involves a two-step determination process [17], (2) cellular mechanisms of Buchnera transmission from maternal bacteriocytes to embryos involve exocytotic and endocytotic cellular processes [16], and (3) maternal and embryonic bacteriomes are not equivalent [2, 54, 55]. Here, we extend work elucidating the mechanisms of host/endosymbiont developmental integration in aphids by describing the developmental atlas of AAAP-536 and APC-8904 mRNAs with respect to bacteriocyte specification and Buchnera transmission. In this study, we demonstrated that AAAP-536 and APC-8904 are expressed at the site of
process, the first from nuclei in the posterior syncytium and the second from the nuclei in the central syncytium. In contrast to Braendle et al. [17], who observed development of the second set of bacteriocytes at stage 13, our data suggest that recruitment of nuclei to the second set of bacteriocytes occurs as early as stage 8 of development (Fig. 2b, c). Alternatively, it is possible that AAAP-536 and Dll, the marker used by Braendle et al. [17], are tracking the recruitment of two different sets of cells. By synthesizing the work of Braendle et al. [17] and Koga et al. [16] with the work we present here, we propose that the nuclei recruited at stage 8, defined by AAAP-536 expression, are destined to form bacteriocyte cells, whereas the nuclei recruited at stages 13–15 are destined to form sheath cells (cells that are not occupied by Buchnera with nuclei much smaller than those of bacteriocytes [16, 17]).

While the clear and discrete expression of AAAP-536 and APC-8904 mRNA at the site of Buchnera invasion prior to Buchnera invasion suggests that both genes are involved in Buchnera recruitment to the blastula, the equally clear and discrete expression of AAAP-536 and APC-8904 in the posterior syncytium in association with the bacterial mass throughout embryogenesis suggests that both genes are involved additionally in bacteriocyte development and possibly play roles in regulating the transovarial transmission of Buchnera.

**Amino acid transporters AAAP-536 and APC-8904 do more than move amino acids across membranes**

A handful of Drosophila orthologs of AAAP-536 and APC-8904 have been functionally characterized. On the basis of our observations of AAAP-536 and APC-8904 expression and the functional characterization of Drosophila orthologs, we hypothesize that AAAP-536 and APC-8904 function not to move amino acids across membranes but rather serve as effectors of the endocytic transfer of Buchnera from maternal bacteriocytes to embryos and as nutrient sensors. AAAP-536, the most highly expressed bacteriocyte-expressed amino acid transporter, is orthologous to Drosophila CG8785 [34], a gene whose functionally characterized paralogs include pathetic [24] and polyphemus (CG12943) [33]. Encoding a proton-assisted transporter (PAT) related to the mammalian solute carrier 36 (SLC36) amino acid transporters, pathetic is a well-known mediator of cell growth [24, 27, 31, 56]. Localized to the cell surface and late endosomes and lysosomes (LELs) of Drosophila fat body, pathetic senses extracellular amino acids and activates the target of rapamycin (TOR) signaling pathway [24, 29, 56]. The other functionally characterized CG8785 paralog, polyphemus, encodes a glutamate transporter that functions to maintain immune-related function by modulating phagocytosis of microbial pathogens and controlling bacterial infection. The other functionally characterized paralog, Drosophila CG8785 [34], is a glutamate transporter that, in Drosophila gut cells, controls cell growth and development and regulates synaptic trafficking [35].

**Buchnera invasion ahead of Buchnera transmission from mother to progeny** (Fig. 1f, l, indicated with arrowheads). Following Buchnera invasion, we found that AAAP-536 and APC-8904 expression was not concomitant with the entirety of the bacterial mass, but rather expression of AAAP-536 and APC-8904 overlaps with only the anterior part of the bacterial mass in association with the syncytial nuclei (Fig. 2b, c, f–h). In addition, we typically observed signal from AAAP-536 in the periphery of nuclei outside the bacterial mass (Fig. 2b, c, indicated with arrowheads); remarkably these nuclei localize to the areas that the bacterial mass moves to in the next developmental stage (Figs. 2b–d, 3a, b). Our results are consistent with the observations of Miura et al. [8] that the developmental origin of bacteriocytes involves a two-step determination of the areas that the bacterial mass moves to in the next developmental stage. In contrast to Braendle et al. [17], who observed development of the second set of bacteriocytes at stage 13, our data suggest that recruitment of nuclei to the second set of bacteriocytes occurs as early as stage 8 of development (Fig. 2b, c). Alternatively, it is possible that AAAP-536 and Dll, the marker used by Braendle et al. [17], are tracking the recruitment of two different sets of cells. By synthesizing the work of Braendle et al. [17] and Koga et al. [16] with the work we present here, we propose that the nuclei recruited at stage 8, defined by AAAP-536 expression, are destined to form bacteriocyte cells, whereas the nuclei recruited at stages 13–15 are destined to form sheath cells (cells that are not occupied by Buchnera with nuclei much smaller than those of bacteriocytes [16, 17]).

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bacterial growth during infection [33]. The second most highly expressed bacteriocyte-expressed amino acid transporter, APC-8904, is orthologous to Drosophila slimfast [21, 34] and closely related to the slimfast paralogs minidiscs [30] and genderblind [57]. Drosophila slimfast and minidiscs functions are associated with nutrition and tissue growth involving the TOR signaling pathway [20, 21, 30, 58]. Encoding a glutamate transporter, genderblind functions in the nervous system with mutations in genderblind yielding a homosexual behavior phenotype [25, 57]. In addition to controlling glutamatergic synapse strength, genderblind functions with polyphemus to affect phagocytosis of microbial pathogens [33]. Significantly, taken together, functionally characterized Drosophila transporters related to AAAP-536 and APC-8904 are nutrient sensors that function as upstream mediators that activate the TOR signaling pathway [23]. Remarkably, activation of TOR signaling stimulates bulk endocytic uptake via clathrin-mediated endocytosis [26, 56]. In light of this genetic evidence from Drosophila and the expression of AAAP-536 and APC-8904 in the posterior syncytium, we hypothesize that AAAP-536 and APC-8904 gene products coordinate with the TOR signaling pathway to acquire Buchnera endosymbionts through an endocytic process that results in reacquisition of the symbiosomal membrane at the time of blastula invasion.

**An evolutionary perspective on bacteriocyte formation in insects**

In contrast to the little that is known at a molecular level about the developmental specification of host bacteriocytes, other aspects of host/symbiont biology are becoming clear. It is clear that host/symbiont genome coevolution involves host/symbiont metabolic collaboration within metabolic pathways for amino acid [1, 6, 7, 54, 59–61] and vitamin biosynthesis [62]. It is also clear that while host/symbiont genome coevolution is constrained by host genome content [63–66], it can also involve the acquisition of new genes by lateral gene transfer [55, 63–65, 67, 68], and duplication of existing genes [34, 36–38, 69, 70]. While characterizing patterns of host/symbiont genome coevolution has been facilitated greatly by the recent accumulation of genome data from host/symbiont pairs, it has also been facilitated by studying bacteriocyte transcriptomes [36, 54, 59, 63, 71]. Bacteriocyte cells that house heritable endosymbionts are common among sap-feeding insects [72]. Substantial insights are likely to be gained by systematically studying similarities and differences in the genes and gene families expressed in bacteriocytes within a comparative framework. Further, which gene sets are conserved in their expression across a diversity of sap-feeding insects through development will likely facilitate determination of the functions that modulate development of the bacteriocyte.

The molecular mechanisms of bacteriocyte development were recently documented in the seed bug, Nysius plebeius [73]. Bacteriocyte cells of N. plebeius differentiate under regulation of homeobox transcription factors that include Ultrabithorax (Ubx), abdominal-A (abd-A), and Antennapedia (Antp). In gene knockdown experiments, suppression of Ubx results in the disappearance of bacteriocyte cells and failure of symbionts to localize, while suppression of Abd-A alters the spatial organization of bacteriocyte cells, and suppression of Antp results in mislocalization of bacteriomes [73]. Notably, homeobox transcription factors that include Ubx/Abd-A, Distal-less (Dll), and Engrailed (En) are also expressed in aphid bacteriocytes [17]. While it is known that Dll is involved in aphid bacteriocyte determination [17], the way that Dll and other homeobox transcription factors determine bacteriocyte formation and bacteriome organization in aphids remains an open question.

**Conclusions**

We are motivated to understand the metabolic and developmental integration of hosts and symbionts in order to understand host/symbiont coevolution. We selected the two amino acid transporters that are most highly expressed in A. pisum bacteriocytes and applied whole-mount in situ hybridization to analyze their developmental expression patterns. While we conjecture that AAAP-536 and APC-8904 gene products are integral to bacteriocyte development, we think that the most intriguing implication of this work concerns our inference that AAAP-536 and APC-8904 gene products modulate TOR signaling. Interpreting our data with knowledge that blastula acquire Buchnera by endocytosis [16] leads us to venture that AAAP-536 and APC-8904 stimulate the endocytic process by coordinating with the TOR signaling pathway to faithfully facilitate the vertical transmission of Buchnera from mother to embryo across tens of millions of years of evolution. Notable, within an evolutionary framework, is the fact that the orthologs of AAAP-536 and APC-8904 show the same patterns of bacteriocyte-enriched expression in the green peach aphid, Myzus persicae [36]. Thus, we speculate that Mper-AAP11 and Mper-APC08 [36] are similarly expressed during asexual embryogenesis in M. persicae. This study highlights that duplicated amino acid transporters have potentially been retained in the aphid genome to play novel developmental roles in symbiont recruitment and genesis of the aphid bacteriome.
Additional files

Additional file 1: Table S1. Sample numbers for the AAAP-536 in situ hybridization experiments by developmental stage.

Additional file 2: Table S2. Sample numbers for the APC-8904 in situ hybridization experiments by developmental stage.

Additional file 3: Table S3. Real-time PCR primers for AAAP-536, APC-8904, and control genes.

Additional file 4: Figure S1. DAPI counterstain of A. pisum asexual embryo at stage 6. The same stage 6 embryo as shown in Fig. 1f. Dashed lines indicate the location of the germ cells. (a) AAAP-536 signal detected with NBT/BCIP brown precipitates and (b) DNA staining of the preparation in (a). No endsymbiotic bacterial DNA signals were detected, confirming that AAAP-536 is expressed before Buchnera invasion. Scale bars are all 20 μm. Abbreviations: ps, posterior syncytium; efic, enlarged follicle cells.

Additional file 5: Figure S2. Locations of Buchnera during asexual A. pisum embryogenesis. Confocal images of 19 developmental stages of asexual A. pisum embryogenesis are shown. DAPI staining labels the location of the germ cells. Morphological characteristics of developmental stages are according to Mura et al. [8]. Scale bars are all 20 μm. Abbreviations: am, amnion; bc, Buchnera symbionts; cl, cephalic lobe; cn, central nervous system; cs, central syncytium; dn, dividing nucleus; efic, enlarged follicle cells; fc, follicle cells; g, gut; gc, germ cells; gl, germarial lumen; gm, germarium; hs, head; ig, invaginating germband; lg, legs; mn, mandible segment; mx, maxilla segment; nc, nerve cells; o, oocyte; on, oocyte nucleus; pb, polar body; ps, posterior syncytium; s, sersa; sn, syncytial nucleus; st, stage; t1–3, thoracic segments.

Additional file 6: Figure S3. Whole-mount in situ hybridization of AAAP-536 at 60°C. Staged embryos hybridized with DIG-labeled antisense (a–f) and sense riboprobes (g–i) of AAAP-536. Signals were revealed by NBT/BCIP brown precipitates. All embryos are displayed with anterior of the germaria to the left. Arrowheads mark AAAP-536-positive signals in the syncytial nucleus. Dashed lines mark the location of germ cells. Arrow indicates AAAP-536 signal not associated with Buchnera. Open arrowheads indicate signals detected in the follicle cells, and yellow arrowhead marks non-specific signal. For developmental stage, please refer to [8]. (a, b) Embryos before Buchnera invasion; transcripts of AAAP-536 were detected in the posterior syncytium (ps) (arrowheads) in late stage 6 embryos. (c–f) Buchnera invasion starts at stage 7 and some AAAP-536 transcripts were detected outside of the bacterial mass (arrowheads). After stage 11, AAAP-536 transcripts were associated with the localization of endsymbiotic bacteria throughout development. (g–i) Developing embryos hybridized with sense riboprobes. Weak ubiquitous signals were detected in the embryos throughout development. Scale bars are all 20 μm. Abbreviations: bc, Buchnera symbionts; cs, central nervous system; gm, germarium; hs, head; ps, posterior syncytium; st, stage; t1–3, thoracic segments.

Additional file 7: Figure S4. AAAP-536 and APC-8904 gene expression in two A. pisum lineages, NTU and LSR1. Comparison of AAAP-536 and APC-8904 expression between embryos before stage 5 and after stage 6 of development. Set A: germaria, oocytes (st 0–2), and embryos at or before stage 5 of development; set B: embryos at or older than stage 6 of development. Expression of AAAP-536 and APC-8904 in aphid tissues normalized to GAPDH. Bar heights indicate mean of the gene expressions across three biological replicates, and error bars indicate 95% confidence interval estimates of the mean expression. (a) Expression of AAAP-536 and APC-8904 in NTU. Student t test comparing gene expression levels between embryos before stage 5 and embryos after stage 6 are not significant (p value = 0.08 in AAAP-536 and p value = 0.19 in APC-8904). (b) Data for LSR1 lineage were collected using the same sample preparation methods as NTU except that a qScript cDNA SuperMix (Quanta Biosciences) was used for CDNA synthesis, 1x PerfectScript SYBR Green FastMix (Quanta Biosciences) was used for PCR products labeling, and MasterMix epp realplex real-time PCR System (Eppendorf) was used for running samples. Expression of AAAP-536 and APC-8904 in LSR1 in embryos before stage 5 and embryos after stage 6 was not significantly different (Student t test: p value = 0.07 for AAAP-536 and p value = 0.40 for APC-8904).

Abbreviations
AAAP: amino acid/auxin permease family; Abd-A: abdominal-A; Antp: Antennapedia; APC: amino acid/polyamine/organocation family; BCP: S-bromo-4-chloro-3-indolylphosphate; βTUB: β-tubulin; bp: base pairs; DAPI: 4′-6-diamidino-2-phenylindole; DIG: digoxigenin; Dll: Distal-less; EF1α: elongation factor 1-alpha; En: en; en; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; h: hours; LELs: late endosomes and lysosomes; min: minute; NBT: nitroblue tetrazolium; ORF: open reading frame; PAT: proton-dependent amino acid transporter; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; RPL7: ribosomal protein L7; RPL32: ribosomal protein L32; s: seconds; SLC36A: mammalian solute carrier 36; TOF: target of rapamycin; Ubc: Ubiquitin; UTR: untranslated region.

Authors’ contributions
ACCW conceived of the experiments. ACCW, HLL, and CC designed the experiments. HLL performed the experiments. HLL, CC, and ACCW contributed to data analysis and preparation of figures for publication. HILL and ACCW drafted the manuscript. All authors contributed to preparation of the final manuscript. All authors read and approved the final manuscript.

Authors’ information
The confocal and in situ localization work reported here was completed by HLL while she was a fellow of the Taiwanese Ministry of Science and Technology Graduate Student Study Abroad Program in the laboratory of ACCW at the University of Miami. HLL completed her Ph.D. in the laboratory of CC studying developmental plasticity of germline development in the pea aphid. CC is a developmental biologist who is studying oogenesis and embryogenesis in aphids and other insects. ACCW is an evolutionary biologist who is studying the coevolution of plant sap-feeding insects and their obligate intracellular bacterial symbionts. Recent work in Wilson’s group focuses on functional characterization of amino acid transporters in sternorrhyncha insects.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
Materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes.

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