Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte–embryo interface

Ryuichi Koga[a], Xian-Ying Meng[a], Tsutomu Tsuchida[a,b], and Takema Fukatsu[a,1]

[a]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan; and [b]Frontier Research Core for Life Sciences, University of Toyama, Toyama 930-8555, Japan

Edited by Nancy A. Moran, Yale University, West Haven, CT, and approved March 26, 2012 (received for review November 23, 2011)

Many insects are associated with obligate symbiotic bacteria, which are localized in specialized cells called bacteriocytes, vertically transmitted through host generations via ovarial passage, and essential for growth and reproduction of their hosts. Although vertical transmission is pivotal for maintenance of such intimate host–symbiont associations, molecular and cellular mechanisms underlying the process are largely unknown. Here we report a cellular mechanism for vertical transmission of the obligate symbiont *Buchnera* in the pea aphid *Acyrthosiphon pisum*. In the aphid body, *Buchnera* cells are transmitted from maternal bacteriocytes to adjacent blastulae at the ovariole tips in a highly coordinated manner. By making use of symbiont-manipulated strains of *A. pisum*, we demonstrated that the facultative symbiont *Serratia* is, unlike *Buchnera*, not transmitted from maternal bacteriocytes to blastulae, suggesting a specific mechanism for *Buchnera* transmission. EM observations revealed a series of exo-/endocytotic processes operating at the bacteriocyte–blastula interface: *Buchnera* cells are exocytosed from the maternal bacteriocyte, temporarily released to the extracellular space, and endocytosed by the posterior syncytial cytoplasm of the blastula. These results suggest that the selective *Buchnera* transmission is likely attributable to *Buchnera*-specific exocytosis by the maternal bacteriocyte, whereas both *Buchnera* and *Serratia* are nonselectively incorporated by the endocytotic activity of the posterior region of the blastula. The sophisticated cellular mechanism for vertical transmission of *Buchnera* must have evolved to ensure the obligate host–symbiont association, whereas facultative symbionts like *Serratia* may coopt the endocytotic component of the mechanism for their entry into the host embryos.

Buchnera aphidicola | endocytosis | *Serratia symbiotica*

Many insects harbor endosymbiotic bacteria in their cells and tissues (1). Facultative symbionts like *Wolbachia pipientis* in diverse insects are of parasitic or conditionally beneficial nature, tend to cause negative effects on their hosts, and exhibit a broad cellular/tissue tropism (2, 3). Meanwhile, obligate symbionts like *Buchnera aphidicola* in aphids are of mutualistic nature, contribute to the fitness of their hosts, and are localized in specialized cells called bacteriocytes (4, 5). In general, these insect symbionts are stably maintained through host generations by vertical transmission from mothers to their offspring (1, 6). Vertical transmission is pivotal for maintenance of such host–symbiont associations, but our understanding of molecular and cellular mechanisms underlying the process is quite limited. In the well-studied facultative symbiotic association of the *Drosophila–Wolbachia* endosymbiosis (2), it has been shown that recognition of stem cell niches and association with dynein/kinesin/microtubule are important for symbiont transmission to host germline and symbiont segregation to host daughter cells (7–9). On the contrary, in the aphid–*Buchnera* endosymbiosis as the model obligate symbiotic association with host and symbiont genomic data available (10, 11), symbiont transmission mechanisms have been poorly understood except for some morphological/cytological aspects. There are a number of microscopic descriptions of the symbiont transmission process in various aphids: some have reported that symbionts circulating in hemolymph are transmitted to a posterior region of the blastula with enlarged follicle cells called "follicle pegs," whereas others described that symbionts are transmitted directly from a neighboring bacteriocyte to the follicular region (reviewed in ref. 1). Recent studies that used specific molecular markers and modern microscopy have provided clearer pictures of the symbiont transmission process in the pea aphid *Acyrthosiphon pisum* (12–14). Based on immunohistochemistry against a symbiont protein, Wilkinson et al. (12) described "a stream of bacteria passing from a single maternal bacteriocyte to the recipient embryo, possibly via a membranous conduit." Meanwhile, based on sophisticated confocal imaging, Miura et al. (13) stated that "the transfer of the bacteria appears to first involve the fusion of a membrane-bound maternal bacterial package with the follicular epithelium in the region of the enlarged posterior follicle cells. A channel between these enlarged follicle cells then appears, and the bacteria flow into the posterior of the embryo." These previous results are summarized into the following hypotheses: the "free symbiont infection" hypothesis (Fig. 1*A*) (1), the "membranous conduit formation" hypothesis (Fig. 1*B*) (1, 12), and the "symbiont packet fusion" hypothesis (Fig. 1*C*) (13).

In addition to the obligate symbiont *Buchnera*, *A. pisum* may be associated with an array of facultative symbionts such as *Serratia symbiotica*, *Hamiltonella defensa*, and *Regiella insecticola*, which are not essential but conditionally beneficial for the host depending on ecological contexts (3, 15). For vertical transmission processes of these facultative symbionts, previous histological descriptions are fragmentary, providing no coherent picture (1, 3).

Here, by making use of symbiont-manipulated strains of *A. pisum* whose infections with *Buchnera* and *Serratia* were experimentally manipulated under specific host genotypes, we demonstrate that a cellular mechanism is operating at the bacteriocyte–embryo interface. The mechanism selectively transports the obligate symbiont *Buchnera*, but not the facultative symbiont *Serratia*, from a maternal bacteriocyte to an adjacent blastula. We also describe the intricate segregation processes of the obligate symbiont and the facultative symbiont during bacteriocyte differentiation in aphid embryogenesis. We suggest that these cellular mechanisms might have evolved to ensure the obligate

Author contributions: R.K. and T.F. designed research; R.K., X.-Y.M., and T.T. performed research; R.K. analyzed data; and R.K. and T.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: t-fukatsu@aist.go.jp.

See Author Summary on page 7597 (volume 109, number 20).

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1119212109/-/DCSupplemental.

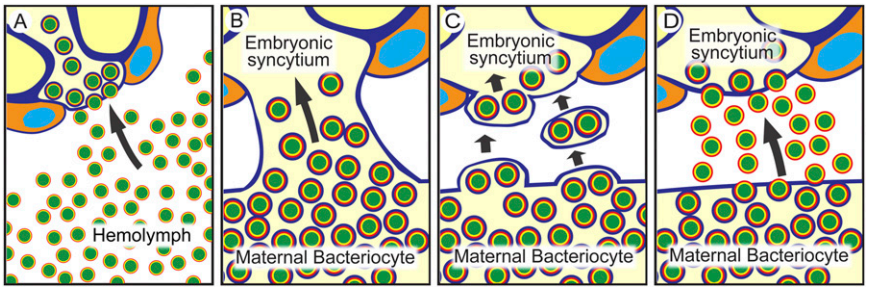

Fig. 1. Hypotheses on the vertical transmission mechanism of the obligate aphid symbiont *Buchnera* from a maternal bacteriocyte to an early embryo: (*A*) free symbiont infection, (*B*) membranous conduit formation, (*C*) symbiont packet fusion, and (*D*) exo-/endocytotic transport.

host–symbiont association, and propose an "exo-/endocytotic transport" hypothesis (Fig. 1*D*) for vertical transmission of *Buchnera* in *A. pisum*.

Results and Discussion

Aphid Strains with Different Symbiont Infections. In this study, we used the following *A. pisum* strains: (*i*) the naturally disymbiotic strain IS, wherein localizations of *Buchnera* and *Serratia* are under normal control, the former in primary bacteriocytes and the latter in secondary bacteriocytes, sheath cells, and hemolymph (Fig. S1*A*); (*ii*) the naturally monosymbiotic strain AIST (named for the National Institute of Advanced Industrial Science and Technology) with *Buchnera* only (Fig. S1*B*); (*iii*) the artificial disymbiotic strain AIST^{IS} generated by microinjection of IS hemolymph into an AIST insect, wherein *Serratia* exhibits a disordered localization, massively proliferating in hemolymph, often invading primary bacteriocytes and coexisting with *Buchnera* therein (Fig. S1*C*); and (*iv*) an artificial monosymbiotic strain AIST$^{IS/rif}$ with *Serratia* only, wherein only *Serratia* is present in bacteriocytes and hemolymph (Fig. S1*D*). In the absence of the obligate symbiont *Buchnera*, AIST$^{IS/rif}$ insects show smaller body size, retarded growth, and reduced fecundity, but manage to survive and reproduce in the presence of the facultative symbiont *Serratia* (16). Table 1 summarizes the attributes of aphid strains used in this study. Here we note that the ordered symbiont localization in the naturally disymbiotic strain, the disordered symbiont localization in the artificial disymbiotic strain, and the bacteriocyte localization of *Serratia* in the artificial monosymbiotic strain are commonly observed in *A. pisum* strains of different geographic origins and genetic backgrounds (16, 17).

Infection Dynamics of *Buchnera* and *Serratia* During Host Development. Miura et al. (13) and Braendle et al. (14) provided detailed histological descriptions of developmental staging, endosymbiont localization, and bacteriocyte formation during parthenogenetic embryogenesis in an American monosymbiotic strain of *A. pisum* infected with *Buchnera* only. By using whole-mount in situ hybridization and confocal imaging (18), we examined a Japanese monosymbiotic strain AIST infected with *Buchnera* only, and confirmed the previous reports. Furthermore, we performed a similar detailed histological inspection of parthenogenetic embryogenesis in a naturally disymbiotic strain IS infected with *Buchnera* and *Serratia*. In the following sections, we adopt the developmental staging reported by Miura et al. (13).

Symbiont Cotransmission. As reported previously (13, 14), symbiont transmission occurred in each blastula at the stage 7, wherein a population of *Buchnera* cells flowed into the embryo via a posterior passage and colonized a syncytium with several large nuclei at the center of the blastula (Fig. 2*A*). In addition to the majority of *Buchnera* cells, a much smaller number of *Serratia* cells also gained entry into the central syncytium (Fig. 2*A*, *Inset*).

Bacteriocyte Cellularization and Symbiont Segregation. During stages 8 to 11, the germ band invaginated and extended into the embryo from the posterior side, pushing the symbiont-containing syncytium toward an anterior region (Fig. 2*B*). From late stage 10 into stage 11, cell membranes appeared in the syncytium, forming uninucleate bacteriocytes. Notably, although *Buchnera* cells and *Serratia* cells had coexisted in the same syncytial cytoplasm by these stages, symbiont segregation occurred in parallel with the bacteriocyte formation: as the cellularization proceeded, *Serratia* signals disappeared in the cellularized areas (Fig. 2*C*). By late stage 12 of embryonic segmentation, bacteriocyte formation as well as symbiont segregation completed: *Buchnera* cells were restricted in uninucleate, so-called "primary" bacteriocytes, whereas *Serratia* cells remained in interstitial areas between the primary bacteriocytes (Fig. 2*D*). The mechanism of the symbiont segregation is unknown, but, although speculative, bacteriocyte-specific lysozyme/lysosomal activities might be involved in the symbiont sorting (19, 20).

Establishment of Primary and Secondary Bacteriocytes. By stage 13 of embryonic limb bud initiation, several large nuclei became evident in the *Serratia*-infected cytoplasmic areas (Fig. 2*E*). Careful confocal imaging revealed continuity of the *Serratia*-harboring cytoplasm, constituting a syncytial "secondary" bacteriocyte located between the primary bacteriocytes (Fig. 2*F*). After the germ band extension (stage 14) and the embryonic flip (or katatrepsis; stage 15), the postflip embryo (stage 16) was positioned with the head anteriorly and with the posterior germ band folded dorsally. Within the folded germ band, the uninucleate primary bacteriocytes and the syncytial secondary bacter-

Table 1. Aphid strains used in this study

Strain	Origin	Symbionts	Symbiont localization
IS	Natural	*Buchnera*, *Serratia*	Ordered and segregated; *Buchnera* in primary bacteriocytes; *Serratia* in secondary bacteriocytes, sheath cells and hemolymph
AIST	Natural	*Buchnera* only	Ordered; *Buchnera* in primary bacteriocytes
AIST^{IS}	Manipulated	*Buchnera*, *Serratia*	Disordered and mixed; not only *Buchnera* but also *Serratia* in primary bacteriocytes
AIST$^{IS/rif}$	Manipulated	*Serratia* only	Disordered; *Serratia* in primary bacteriocytes and hemolymph; fitness severely damaged

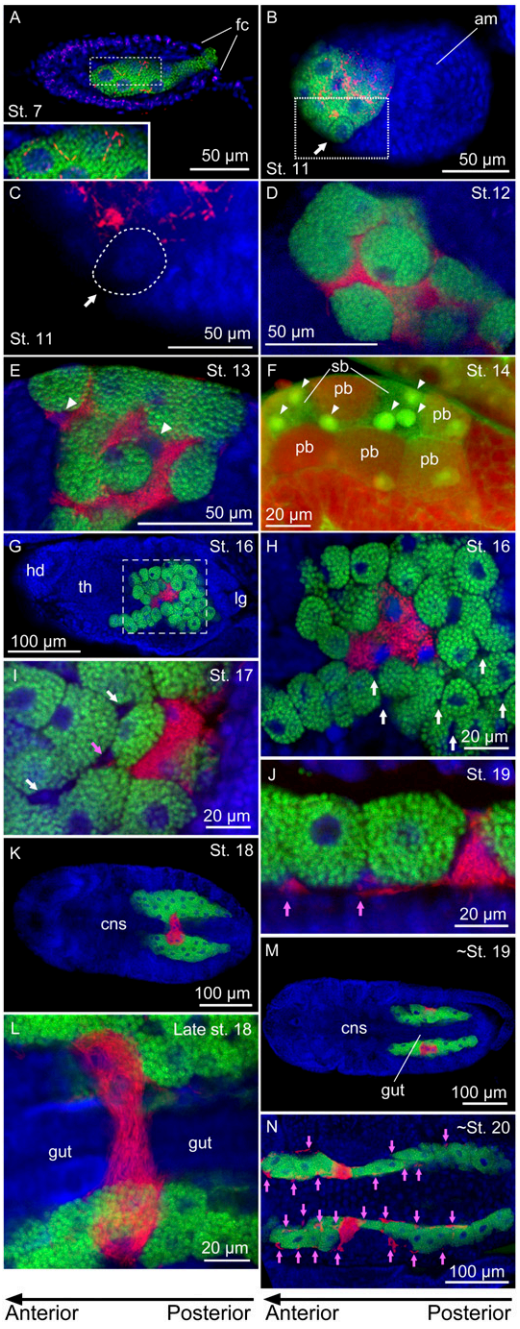

Fig. 2. Infection dynamics of *Buchnera* and *Serratia* during embryonic development in the strain IS of *A. pisum*. In the fluorescent microscopic images, green, red, and blue signals indicate *Buchnera* cells, *Serratia* cells, and host insect nuclei, respectively, unless otherwise indicated. Left and right of each panel are anterior and posterior sides, respectively. (*A*) A stage 7 embryo, to which *Buchnera* and *Serratia* are infecting via a posterior passage. *Inset*: Enlarged image of the dotted rectangle, wherein *Serratia* cells are clearly seen in addition to *Buchnera* cells in the central syncytium. (*B*) A stage 11 embryo in which the symbiont-infected syncytium is cellularizing. Arrow indicates a bacteriocyte that has almost completed the cellularization. (*C*) An enlarged image of *Serratia* distribution in the dotted rectangle of *B*, from which *Buchnera* signals are removed. Note that no *Serratia* signals are detected in the completed bacteriocyte (dotted area indicated by an arrow). (*D*) A late stage 12 embryo in which bacteriocyte formation and symbiont segregation have completed. (*E*) A stage 13 embryo in which multiple nuclei of the secondary bacteriocyte become evident (arrowheads). (*F*) A stage 13 embryo stained with green TOTO-3 and red Alexa Fluor 488 phalloidin in which the multiple nuclei (arrowheads) are clearly seen in a large and pleomorphic cytoplasm of the secondary bacteriocyte. (*G*) A stage 16 embryo in which uninucleate primary bacteriocytes harboring *Buchnera* and a syncytial secondary bacteriocyte harboring *Serratia* constitute a large bacteriome. (*H*) An enlarged image of the bacteriome in the dotted rectangle of *G* in which a number of small nuclei of sheath cells (white arrows) are seen. (*I*) The bacteriome of a stage 17 embryo in which *Serratia* signals appear in the sheath cells (pink arrow). (*J*) The bacteriome of a stage 19 embryo in which the sheath cells are densely populated by *Serratia* cells (pink arrows). (*K*) A stage 18 embryo, whose bacteriome is laterally separating at the region of the secondary bacteriocyte. (*L*) The bacteriome bridge of a late stage 18 embryo about to be torn apart. (*M*) A stage 19 embryo in which a pair of syncytial secondary bacteriocytes are established in the bacteriomes. (*N*) The bacteriomes of a stage 20 embryo. *Buchnera* is localized in a number of primary bacteriocytes, whereas *Serratia* is found in a pair of secondary bacteriocytes and many tiny sheath cells (pink arrows). am, amnion; cns, central nervous system; emb, embryo; fc, follicle cell; hd, head; pb, primary bacteriocyte; sb, secondary bacteriocyte; th, thorax.

iocyte formed a conspicuous symbiotic organ, or "bacteriome," which was nestled on the dorsal side of the embryo (Fig. 2*G*).

Appearance of Sheath Cells. At stage 16, a number of small cell nuclei became recognizable between the bacteriocytes within the bacteriome (Fig. 2*H*, white arrows). By stage 17 of germ band retraction, *Serratia* signals appeared in these small cells (Fig. 2*I*, pink arrow). Judging from cytologic findings and *Serratia* localization, these cells are "sheath cells" specialized for harboring facultative bacterial symbionts in aphids (15, 16, 21). By stage 18 of eye differentiation, the sheath cells were densely populated by *Serratia* cells (Fig. 2*J*, pink arrows). By using anti–*Distal-less* (*Dll*) antibody as molecular marker of bacteriocyte differentiation, Braendle et al. (14) identified a second population of bacteriocytes in the aphid embryogenesis: at stage 13, 40 to 60 *Dll*-expressing cells appear near the posterior end of the dorsal germ band region; during stages 14 and 15, these cells migrate to the region of the original bacteriocytes; and at stage 16, the smaller cells intercalate between the larger original bacteriocytes (14). We suggest that the second bacteriocyte population might correspond to the sheath cells, although this hypothesis should be verified by using a molecular marker that can specifically label the sheath cells.

Dynamic Rearrangement of Bacteriome and Fission of Secondary Bacteriocytes. In late embryogenesis during stages 16 to 19, dynamic topological rearrangement of the bacteriome occurred. At the stage 16, the bacteriome was coherent, located between the embryonic dorsum and the folded posterior germ band (Fig. 2*G* and Figs. S2*A* and S3 *A–F*). At stage 17, as germ band retraction and dorsal closure of the embryonic body proceeded, the gut tube was located dorsally on the bacteriome while both sides of the bacteriome moved upward in a rotating manner (Figs. S2*B* and S3 *G–J*). At stage 18, the lateral upward rotation of the bacteriome further proceeded, so the bacteriome was torn off at the ventral side, where the syncytial secondary bacteriocyte formed a "bacteriome bridge" connecting the separating halves of the bacteriome (Fig. 2 *K* and *L* and Figs. S2*C* and S3 *K–Q*). At stage 19, the left and right halves of the bacteriome reassociated on the dorsal side, and a pair of syncytial secondary bacteriocytes were formed on the ventral side (Fig. 2 *M* and *N* and Fig. S2*D*). In this way, all the cellular components of the bacteriome, namely the primary bacteriocytes harboring *Buchnera*, and the secondary bacteriocytes and the sheath cells harboring *Serratia* (and potentially other facultative symbionts; ref. 15), were established and completed in the parthenogenetic embryogenesis of *A. pisum* (Movie S1).

Association of Blastulae with Maternal Bacteriocytes for Symbiont Transmission. Among the developmental processes described here, the onset of the symbiotic association at the blastula stage (or

stage 7; Fig. 2*A*) was of particular interest. Hence, we quenched autofluorescence of whole nymphal insects by a hydrogen peroxide treatment (18), performed whole-body in situ hybridization to preserve tissue integrity, and observed their embryos and bacteriocytes through transparent cuticles under a confocal microscope. The blastulae were located in the tip region of the ovarioles, and the majority of them were found adjacent to maternal bacteriocytes. Typically, the posterior side of each blastula was closely associated with a maternal bacteriocyte. At the attachment site, *Buchnera* signals in the embryos looked continuous with *Buchnera* signals in the associated maternal bacteriocytes (Fig. 3 *A*–*C*).

Selective Transmission of *Buchnera* at Bacteriocyte–Blastula Interface. Similar histological inspection of the artificial disymbiotic strain AIST^{IS} unveiled an unexpected phenomenon involved in *Buchnera* transmission at the bacteriocyte–blastula interface. In strain AIST^{IS}, localization of *Serratia* is often strikingly disordered, massively proliferating in the hemolymph and invading the primary bacteriocytes that normally harbor *Buchnera* only, which may reflect uncontrolled or virulent behavior of the transfected facultative symbiont (16, 17). In the AIST^{IS} insects, the blastulae were also found in the tip region of the ovarioles, and most of them were associated with maternal bacteriocytes. Interestingly, whereas the maternal bacteriocytes were coinfected with *Buchnera* and *Serratia*, all the blastulae were preferentially infected with *Buchnera*, with few *Serratia* signals therein (Fig. 4*A*). At the attachment site, a striking discontinuity was observed: although both *Buchnera* and *Serratia* signals were present in the maternal bacteriocyte, almost exclusively *Buchnera* was found in the blastulae (Fig. 4 *A*–*C*). These observations clearly refuted the presence of membrane conduit between the maternal bacteriocyte and the blastula, and suggested an unknown mechanism for selective *Buchnera* transmission at the bacteriocyte–blastula interface.

***Buchnera*-Independent *Serratia* Transmission to Blastulae.** In the artificial disymbiotic strain AIST^{IS}, *Serratia* cells in the maternal bacteriocytes were not transferred to the blastulae (Fig. 4 *A*–*C*). In the naturally disymbiotic strain IS, although the maternal bacteriocytes were *Serratia*-free, *Serratia* signals were found in the blastulae (Fig. 3 *B* and *C*). These observations suggested that not the maternal bacteriocyte but the surrounding hemolymph should be the source of *Serratia* inoculum to blastulae. During the embryogenesis of the artificial monosymbiotic strain AIST$^{IS/rif}$ infected with *Serratia* only, the facultative symbiont *Serratia* behaved as if it replaced the symbiotic niche of the obligate symbiont *Buchnera*, infecting the uninucleate primary bacteriocytes and forming a *Serratia*-occupied bacteriome (Fig. 5 *A*–*D*). In the AIST$^{IS/rif}$ insects, few blastulae were found associated with the *Serratia*-infected maternal bacteriocytes, but *Serratia* transmission

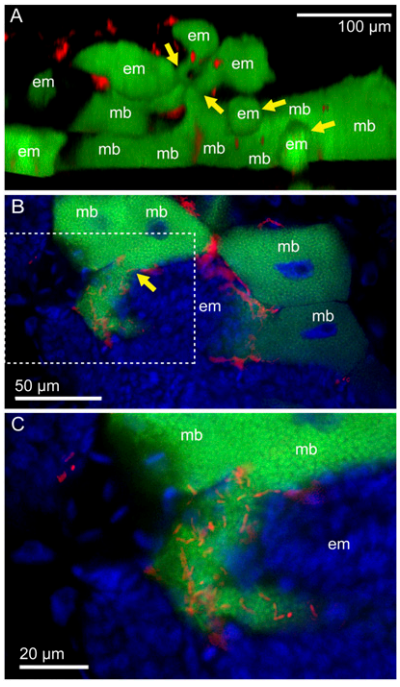

Fig. 3. Vertical transmission of *Buchnera* and *Serratia* to blastulae in the strain IS of *A. pisum*. (*A*) Projection image of the bacteriome of a 3-d-old nymph constructed by serial confocal optical sections. Many embryos (em) and maternal bacteriocytes (mb) are seen. Several blastulae are associated with maternal bacteriocytes in the process of symbiont transmission. Yellow arrows indicate the junction sites between blastulae and maternal bacteriocytes. (*B*) A confocal section of a blastula and maternal bacteriocytes. (*C*) An enlarged image of the dotted box in *B*. Green, red, and blue signals indicate *Buchnera*, *Serratia*, and host nuclei, respectively. Note that blue signals of host nuclei are not shown in *A*.

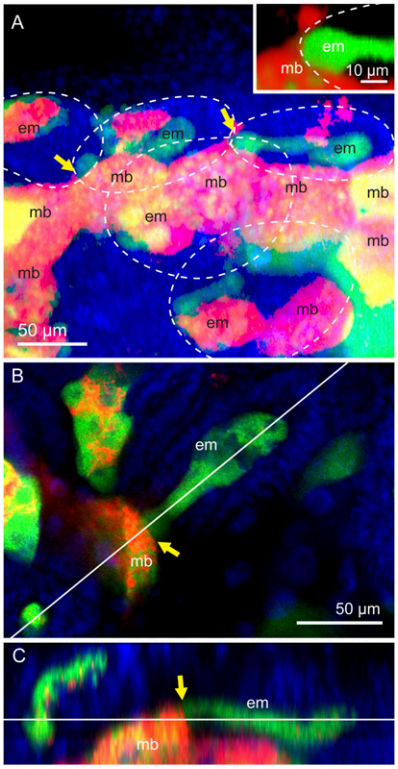

Fig. 4. Selective *Buchnera* transmission at the bacteriocyte–blastula interface observed in the strain AIST^{IS} of *A. pisum*. (*A*) Projection image of the bacteriome of a 3-d-old nymph constructed by serial confocal optical sections. All maternal bacteriocytes (mb) are coinfected with *Buchnera* (green) and *Serratia* (red). Several blastulae are associated with maternal bacteriocytes in the process of symbiont transmission (yellow arrows). Note that the embryo contains substantially *Buchnera* signals only, whereas the maternal bacteriocyte exhibits strong *Serratia* signals in addition to *Buchnera* signals. *Inset*: Highlighted blastula–bacteriocyte junction. Contour of embryos is shown by white dotted circles. (*B*) A confocal image of another blastula in the process of symbiont transmission. This section corresponds to the plane indicated by a horizontal line in *C*. (*C*) A *z*-axis image constructed from serial confocal sections of the area shown in *B*. This section corresponds to the plane indicated by a diagonal line in *B*. Green, red, and blue signals indicate *Buchnera*, *Serratia*, and host nuclei, respectively. Yellow arrows show blastula–bacteriocyte junctions.

via the posterior passage was consistently observed (Fig. 5*A*), reinforcing the hypothesis that not the maternal bacteriocyte but the surrounding hemolymph is the source of *Serratia* inoculum. These observations indicated that *Serratia* transmission can proceed independently of *Buchnera* transmission, suggesting different transmission mechanisms for the obligate symbiont and the facultative symbiont.

EM of Bacteriocyte–Blastula Interface. What transmission processes are observed at the interface between the maternal bacteriocytes and the blastulae in *A. pisum*? Our detailed histological inspections revealed that mature embryos at stage 20 within maternal overioles already contained a few blastulae (i.e., the "telescoping aphid generations"), and vertical symbiont transmission was often taking place in these granddaughter embryos. We carefully dissected and collected mature embryos from naturally disymbiotic IS insects, cautiously fixed and embedded them, and processed the samples into serial ultrathin sections for transmission EM. After exhaustive trials and surveys, we finally obtained good preparations of blastulae wherein the symbiont transmission was taking place and the bacteriocyte–embryo interface was preserved without damage.

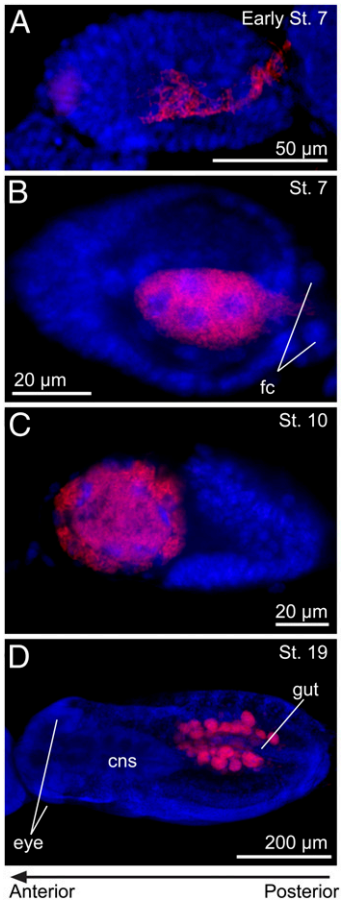

Fig. 5. Vertical transmission and localization of *Serratia* in the *Buchnera*-eliminated strain AIST$^{IS/rif}$ of *A. pisum*. (*A*) An early stage 7 blastula in the process of *Serratia* transmission via the posterior passage. (*B*) A stage 7 blastula whose central syncytium is filled with *Serratia* cells. (*C*) A stage 10 embryo in which the *Serratia*-infected syncytium is located anteriorly. (*D*) A stage 19 embryo in which a pair of bacteriome lobes harboring *Serratia* are located in the abdomen. Red and blue signals indicate *Serratia* and host nuclei, respectively. cns, central nervous system; fc, follicle cell.

Junction Structure Between Maternal Bacteriocyte and Blastula. Fig. 6*A* shows the posterior region of a blastula in close association with maternal bacteriocytes, where the symbiont transmission is ongoing. Despite the close spatial proximity, no cytoplasmic connection was present between the bacteriocyte and the embryo. No structure reminiscent of a cytoplasmic packet was observed. Between the bacteriocyte and the embryo, a narrow but distinct extracellular space did always exist. The main body of the blastula consisted of a surface cell layer and an inner syncytial cytoplasm that contained several polyploid presumptive bacteriocyte nuclei and many *Buchnera* and *Serratia* cells. Notably, a specialized cytological configuration was observed at the posterior embryonic region: highly enlarged polyploid follicle cells were located at the posterior pole of the blastula, surrounding the symbiont passage and forming a transmission apparatus. This structure probably corresponds to a follicle peg (1) or "enlarged follicle cells" (13) identified in previous light microscopic studies. Strikingly, specifically in the space between the transmission apparatus and the maternal bacteriocyte, many *Buchnera* and *Serratia* cells were freely present extracellularly (Fig. 6*A*). In the other regions around the maternal bacteriocytes and the blastulae, no *Buchnera* cells and only a few *Serratia* cells were found extracellularly. These cytological traits were consistently observed in multiple blastulae in the process of symbiont transmission. Hereafter, we designate the area between the transmission apparatus and the maternal bacteriocyte as the transmission center, and describe its fine structure and cytological details.

Transmission Process of *Buchnera*. Specifically on the surface of the maternal bacteriocyte at the transmission center, many *Buchnera* cells were extruding to the extracellular space, presumably representing the process of exocytotic release (Fig. 6*B*). No other location on the surface of the maternal bacteriocytes exhibited such a peculiar cytological trait. On the surface of the transmission center of the embryonic side, strikingly, a number of thin cytoplasmic extensions were protruding to the extracellular space, by which free *Buchnera* cells were trapped (Fig. 6*C*) and engulfed into the cytoplasmic passage toward the inner syncytial cytoplasm of the blastula (Fig. 6*D*). Close examination of the membrane structures surrounding the *Buchnera* cells confirmed that exocytotic and endocytotic processes were involved in the symbiont transmission from the bacteriocyte to the embryo: endocellular *Buchnera* cells in the maternal bacteriocyte exhibited a three-layered membrane structure (Fig. 6*E*); extracellular *Buchnera* cells showed a two-layered membrane structure, losing the outermost host-derived membrane (Fig. 6 *F* and *G*); and endocellular *Buchnera* cells engulfed by the transmission apparatus toward the blastula showed a restored three-layered membrane structure (Fig. 6 *H* and *I*).

Exocytotic and Endocytotic Mechanisms Involved in Selective *Buchnera* Transmission at Bacteriocyte–Blastula Interface. These results indicate that (*i*) vertical transmission of *Buchnera* occurs specifically at the bacteriocyte–blastula interface, or the transmission center, in a highly regulated manner; (*ii*) at the transmission center, *Buchnera* cells are exocytosed from the maternal bacteriocyte, temporarily released to the extracellular space, and endocytosed by the cytoplasm of the embryonic syncytium surrounded by enlarged follicle cells; and (*iii*) no cytoplasmic connection is formed between the maternal bacteriocyte and the blastula. Considering that both *Buchnera* and *Serratia* cells are endocytosed by the embryonic syncytium (Fig. 6*A*), the selective *Buchnera* transmission in AIST^{IS} insects (Fig. 4 *A*–*C*) is likely attributable to *Buchnera*-specific exocytosis by the maternal bacteriocyte at the transmission center.

Contrasting Hypotheses on *Buchnera* Transmission Mechanism. Based on these results, we propose the exo-/endocytotic transport

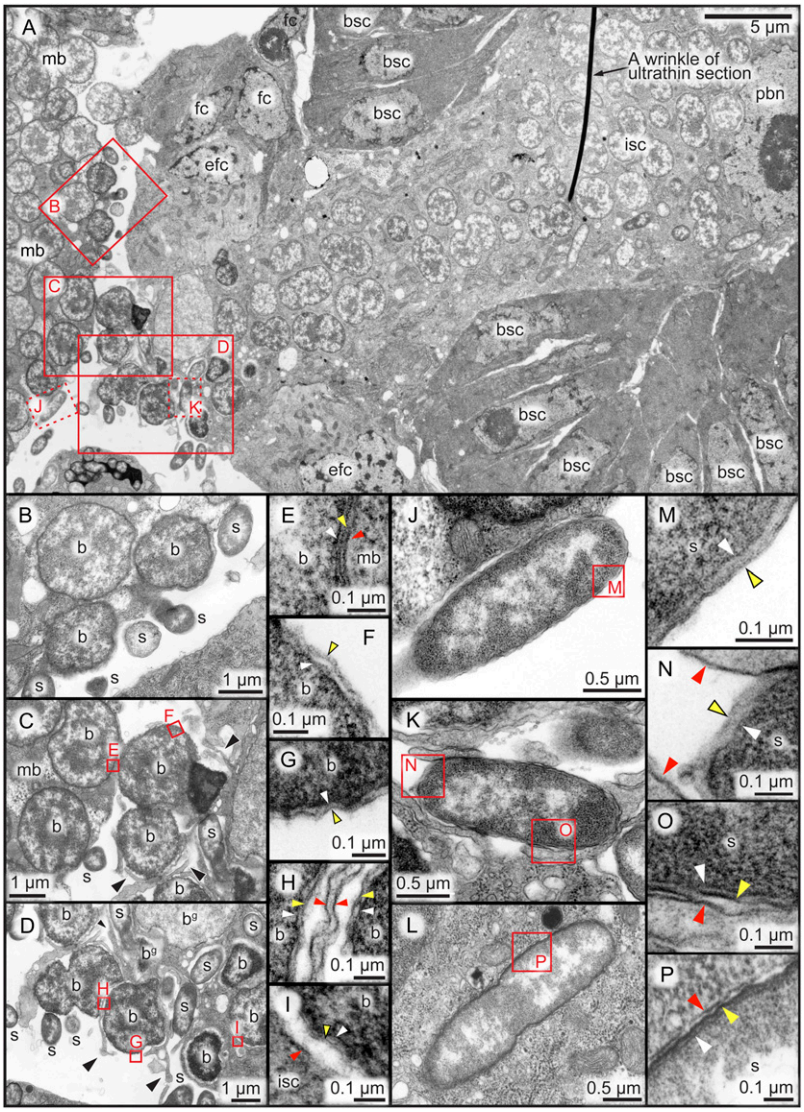

Fig. 6. Transmission EM images of symbiont transmission processes at the bacteriocyte–blastula interface in the IS strain of *A. pisum*. (*A*) A low-magnification image of the posterior region of a blastula adjacent to a maternal bacteriocyte, where the symbiont transmission is ongoing. Red rectangles correspond to the locations of the following magnified images. (*B*) *Buchnera* cells protruding from the surface of a maternal bacteriocyte at the transmission center. (*C*) Extracellular *Buchnera* cells trapped by thin cytoplasmic extensions (black arrowheads) protruding from the posterior pole of the blastula. (*D*) *Buchnera* cells endocytosed into the inner syncytial cytoplasm of the blastulae. *Serratia* cells are also endocytosed into the syncytial cytoplasm. Some of the *Buchnera* cells look degenerative in the host cytoplasm. (*E*) Three-layered membrane structure of a *Buchnera* cell within the maternal bacteriocyte. White, yellow, and red arrowheads indicate bacterial inner membrane, bacterial outer membrane, and host cell membrane, respectively. (*F*) Two-layered membrane structure of an extracellular *Buchnera* cell. (*G*) Two-layered membrane structure of the extracellular side of a *Buchnera* cell in the process of endocytosis. (*H*) Three-layered membrane structure of the engulfed side of the same *Buchnera* cell. (*I*) Three-layered membrane structure of an endocellular *Buchnera* cell in the embryonic syncytial cytoplasm. (*J*) An extracellular *Serratia* cell in the transmission center. (*K*) A *Serratia* cell in the process of endocytosis. (*L*) A *Serratia* cell within the syncytial cytoplasm of the blastula. (*M*) Two-layered membrane structure of an extracellular *Serratia* cell. (*N*) Two-layered membrane structure of the extracellular side of a *Serratia* cell in the process of endocytosis. (*O*) Three-layered membrane structure of the engulfed side of the same *Serratia* cell. (*P*) Three-layered membrane structure of an endocellular *Serratia* cell in the embryonic syncytial cytoplasm. b, *Buchnera* cell; b^g, presumably degenerating *Buchnera* cell; bsc, blastula surface cell; efc, enlarged follicle cell; fc, follicle cell; isc, inner syncytial cytoplasm; mb, maternal bacteriocyte; pbn, presumptive bacteriocyte nucleus; s, *Serratia* cell.

hypothesis (Fig. 1*D*) for vertical transmission mechanism of *Buchnera* in *A. pisum*. Certainly, we obtained microscopic images that looked like showing bacteriocyte–blastula conduits or packet-fused–like blastulae at the resolution of confocal optics (e.g., Figs. 3 and 4), but our EM observations clearly rejected the membranous conduit formation hypothesis (Fig. 1*B*) (1, 12) and the symbiont packet fusion hypothesis (Fig. 1*C*) (13). At present, we cannot reject the free symbiont infection hypothesis (Fig. 1*A*). Although we did not observe such instances in the mature embryos, disintegrated bacteriocytes and released *Buchnera* cells in the hemocoel are often observed in adult aphids (1), and it is conceivable that such *Buchnera* cells might be incorporated into the blastulae. It should be also kept in mind that we examined only *A. pisum*, and cannot exclude the possibility that different mechanisms for symbiont transmission might be found in different aphid lineages.

Endocytotic *Serratia* Transmission to Blastula. A number of free *Serratia* cells were observed in the extracellular space of the transmission center, whereas no *Serratia* cells were harbored in

and exocytosed from the maternal bacteriocytes (Fig. 6*A*). At the embryonic side of the transmission center, free *Serratia* cells were trapped by cytoplasmic extensions and endocytosed into the transmission passage (Fig. 6 *J–P*), as *Buchnera* cells were incorporated into the blastula (Fig. 6 *C–I*). These results indicate that (*i*) vertical transmission of *Serratia* also occurs at the bacteriocyte–blastula interface; (*ii*) extracellular *Serratia* cells are endocytotically incorporated into the blastula; and (*iii*) unlike *Buchnera* transmission, no spatially regulated exocytotic process is involved in *Serratia* transmission. It seems plausible that the endocytotic mechanism for *Serratia* transmission might be the same as that for *Buchnera* transmission. On account of the facultative nature of the symbiosis, although speculative, *Serratia* might use the preexisting endocytotic mechanism of the blastula whose original role is for vertical transmission of the obligate symbiont *Buchnera*.

Aggregation of *Serratia* Around Ovariole Tips. We identified an interesting in vivo localization of *Serratia* that might be relevant to its targeting to host embryos for vertical transmission. Fig. 7 shows whole-body in situ hybridization of *Buchnera* and *Serratia* in nymphal *A. pisum*. In newborn nymphs, *Serratia* signals were found in the secondary bacteriocytes and the sheath cells in association with *Buchnera* signals in the bacteriome (Fig. 7 *A* and *B*). In older nymphs, extracellular proliferation of *Serratia* became prominent, with particularly intense localization around the ovariole tips (Fig. 7 *C* and *D*, arrows). Confocal imaging of the ovariole tips confirmed aggregation of *Serratia* cells on and around the ovariole tips, the location of blastulae (Fig. 7*E*). Although speculative, these localization patterns might be facilitating encounter and vertical transmission of *Serratia* to blastulae of the host insect.

Possible Cellular and Molecular Mechanisms for Symbiont Transmission. The spatially restricted formation of the transmission center and its peculiar cytological traits are suggestive of intricate molecular and cellular interactions between the maternal bacteriocyte and the blastula. The up-regulated endocytotic activity at the posterior pole of the blastula must be an important mechanism for vertical transmission of the obligate symbiont *Buchnera* and the facultative symbiont *Serratia*. It seems likely that the blastula might somehow induce exocytosis of *Buchnera* cells at an adjacent area of the neighboring maternal bacteriocyte, although it is totally unknown what signals mediate the interactions. Alternatively, it is also conceivable that the neighboring maternal bacteriocyte might be involved in the activated endocytosis at the posterior pole of the blastula. *Buchnera* is allied to the gammaproteobacterial family Enterobacteriaceae, which includes well studied pathogens like *Salmonella*, *Shigella*, and *Yersinia* (22). These pathogens are known for their sophisticated molecular mechanisms for invasion into eukaryotic host cells by delivering effector molecules into the host cytoplasm via the type III secretion system, manipulating the cytoskeletal machinery of the host cell, and facilitating lamellipodia formation, endocytotic trapping, and internalization of bacterial cells (23, 24). In this context, it seems meaningful that, although the *Buchnera* genome lacks orthologues of the type III secretion system genes (10, 25), numerous flagellar basal bodies, which are evolutionarily homologous to the type III secretion system (26), are present on the cell membrane of *Buchnera*, which might mediate molecular transports from and to the host cytoplasm (27).

Conclusion and Perspective. Fig. 8 summarizes the infection cycle of the obligate symbiont *Buchnera* and the facultative symbiont *Serratia* in the parthenogenetic phase of *A. pisum*. In conclusion, we describe a cellular mechanism for vertical transmission of

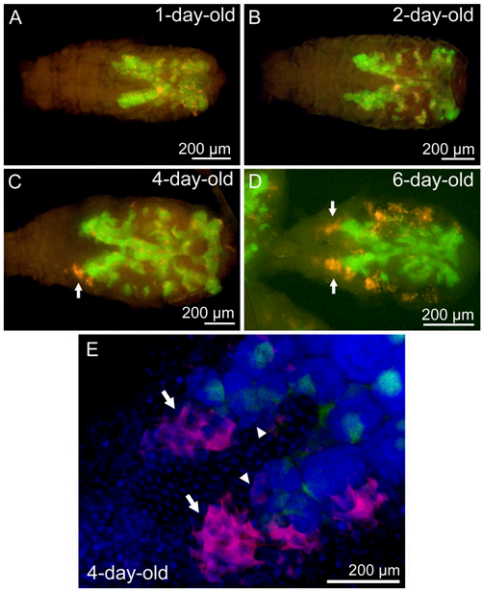

Fig. 7. Localization of *Serratia* and *Buchnera* visualized by whole-body in situ hybridization in the IS strain of *A. pisum*: (*A*) 1-d-old, first instar nymph; (*B*) 2-d-old, second instar nymph; (*C*) 4-d-old, third instar nymph; and (*D*) 6-d-old, fourth instar nymph. (*E*) Ovariole tips of a 4-d-old, third instar nymph. Red, green, and blue signals indicate *Serratia*, *Buchnera*, and host nuclei, respectively. Note that blue signals of host nuclei are not shown in *A–D*. Arrows show the ovariole tips where *Serratia* cells aggregate, whereas arrowheads indicate blastulae in which vertical symbiont transmission occurs.

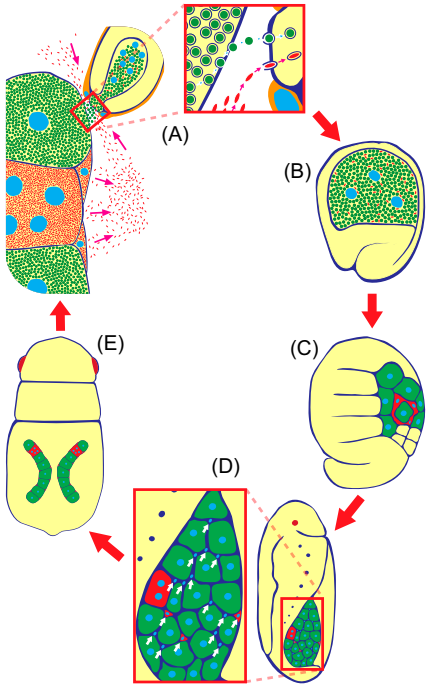

Fig. 8. Infection dynamics of *Buchnera* and *Serratia* in the parthenogenetic life cycle of *A. pisum*. (*A*) Symbiont transmission from maternal bacteriocyte to blastula. (*B*) Formation of symbiont-harboring syncytial cytoplasm. (*C*) Bacteriocyte cellularization and symbiont sorting. (*D*) Appearance of sheath cells. (*E*) Establishment of paired bacteriomes. Green, orange, and yellow indicate *Buchnera*, *Serratia*, and host embryo, respectively.

Buchnera at the bacteriocyte–embryo interface, which must have evolved to ensure the mutualistic association with the obligate symbiont. Also, we suggest the possibility that facultative symbionts like *Serratia* are taking a "free ride" on the preexisting transmission mechanism to gain entry into the host embryos. Bacteriocytes for harboring obligate symbiotic bacteria are found in diverse insect taxa, including the Hemiptera, Coleoptera, Diptera, Phthiraptera, and others, and those symbiotic cells are likely to be of independent evolutionary origins (1, 5). Within the Hemiptera, meanwhile, most of the major taxa such as aphids, coccids, whiteflies, psyllids, leafhoppers, cicadas, and others possess the bacteriocytes in common, but their bacterial symbionts are often quite divergent phylogenetically (4, 5). The commonality and diversity of the symbiotic cells and associated transmission mechanisms in the Hemiptera are of evolutionary interest and to be established in future studies.

Materials and Methods

Insects. Aphid strains IS, AIST, AIST^{IS}, and AIST$^{IS/rif}$ were used in this study (Table 1 and Fig. S1) (16, 17, 21). The insects were reared on seedlings of the broad bean *Vicia faba* at 20 °C in a long-day regimen of 16 h light and 8 h dark.

Cytological Staining. Fixation and staining of dissected ovarioles were performed essentially as described (13). Ovarioles were dissected from second, third, and forth instar nymphs in ice-cold Dulbecco PBS solution (DPBS; Sigma) and fixed in ice-cold 4% (wt/vol) formaldehyde/DPBS for approximately 30 min. DNA was stained with 1 μM TOTO-3 (Molecular Probes), and filamentous actin was visualized with Alexa Fluor 488 phalloidin (Molecular Probes).

In Situ Hybridization. Whole-mount in situ hybridization was performed as described previously (18). Ovarioles were dissected and fixed in Carnoy solution [ethanol:chloroform:acetic acid at 6:3:1 (vol/vol) ratio] overnight. Whole nymphs were fixed in Carnoy solution overnight; their head, legs, antennae, and cornicles were removed by forceps in 80% (vol/vol) ethanol; and their cuticle was pricked throughout the body with a thin needle. Tissue samples were then treated with 6% (wt/vol) hydrogen peroxide in 80% ethanol for several days for quenching autofluorescence of the tissues, thoroughly washed with 100% ethanol, and stored at −20 °C until use. The following oligonucleotide probes were used for in situ hybridization: Cy5-ApisP2a (5′-Cy5-CCT CTT TTG GGT AGA TCC-3′) targeting 16S rRNA of *Buchnera* and Cy3-PASSisR (5′-Cy3-CCC GAC TTT ATC GCT GGC-3′) targeting 16S rRNA of *Serratia*. The tissue samples were hydrated with DPBS containing 0.3% Triton X-100, incubated with hybridization buffer [20 mM Tris-HCl (pH 8.0), 0.9 M NaCl, 0.01% SDS, 30% (vol/vol) formamide] containing 100 nM each of the probes and 0.5 μM SYTOX Green (Molecular Probes) overnight, washed thoroughly with DPBS containing 0.3% Triton X-100, mounted in SlowFade antifade solution (Molecular Probes), and observed under an epifluorescent microscope (Axiophot; Carl Zeiss) and/or a laser scanning microscope (PASCAL5; Carl Zeiss).

EM. Dissected mature embryos were fixed and embedded in Spurr resin as described previously (21). Initially, semi-ultrathin sections were made with an ultramicrotome (Ultracat-N; Leichert-Nissei), mounted on glass slides, stained with toluidine blue, and observed under a light microscope. A sample was scraped and observed little by little, and when a blastula in the process of symbiont transmission was identified, the sample was processed into serial ultrathin sections, mounted on copper meshes, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (model H-7000; Hitachi).

ACKNOWLEDGMENTS. The authors thank S. Koike, J. Makino, and W. Kikuchi for technical and secretarial assistance and Y. Kamagata for logistic support.

1. Buchner P (1965) *Endosymbiosis of Animals with Plant Microorganisms* (Interscience, New York).
2. Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: Master manipulators of invertebrate biology. *Nat Rev Microbiol* 6:741–751.
3. Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu Rev Entomol* 55:247–266.
4. Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu Rev Microbiol* 59:155–189.
5. Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. *Annu Rev Genet* 42:165–190.
6. Bright M, Bulgheresi S (2010) A complex journey: Transmission of microbial symbionts. *Nat Rev Microbiol* 8:218–230.
7. Frydman HM, Li JM, Robson DN, Wieschaus E (2006) Somatic stem cell niche tropism in *Wolbachia*. *Nature* 441:509–512.
8. Serbus LR, Casper-Lindley C, Landmann F, Sullivan W (2008) The genetics and cell biology of *Wolbachia*-host interactions. *Annu Rev Genet* 42:683–707.
9. Fast EM, et al. (2011) *Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche. *Science* 334:990–992.
10. Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H (2000) Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* 407:81–86.
11. International Aphid Genomics Consortium (2010) Genome sequence of the pea aphid *Acyrthosiphon pisum*. *PLoS Biol* 8:e1000313.
12. Wilkinson TL, Fukatsu T, Ishikawa H (2003) Transmission of symbiotic bacteria *Buchnera* to parthenogenetic embryos in the aphid *Acyrthosiphon pisum* (Hemiptera: Aphidoidea). *Arthropod Struct Dev* 32:241–245.
13. Miura T, et al. (2003) A comparison of parthenogenetic and sexual embryogenesis of the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphidoidea). *J Exp Zoolog B Mol Dev Evol* 295:59–81.
14. Braendle C, et al. (2003) Developmental origin and evolution of bacteriocytes in the aphid-*Buchnera* symbiosis. *PLoS Biol* 1:E21.
15. Moran NA, Russell JA, Koga R, Fukatsu T (2005) Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. *Appl Environ Microbiol* 71:3302–3310.
16. Koga R, Tsuchida T, Fukatsu T (2003) Changing partners in an obligate symbiosis: A facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. *Proc Biol Sci* 270:2543–2550.
17. Koga R, Tsuchida T, Sakurai M, Fukatsu T (2007) Selective elimination of aphid endosymbionts: Effects of antibiotic dose and host genotype, and fitness consequences. *FEMS Microbiol Ecol* 60:229–239.
18. Koga R, Tsuchida T, Fukatsu T (2009) Quenching autofluorescence of insect tissues for in situ detection of endosymbionts. *Appl Entomol Zool (Jpn)* 44:281–291.
19. Nakabachi A, et al. (2005) Transcriptome analysis of the aphid bacteriocyte, the symbiotic host cell that harbors an endocellular mutualistic bacterium, *Buchnera*. *Proc Natl Acad Sci USA* 102:5477–5482.
20. Nishikori K, Morioka K, Kubo T, Morioka M (2009) Age- and morph-dependent activation of the lysosomal system and *Buchnera* degradation in aphid endosymbiosis. *J Insect Physiol* 55:351–357.
21. Fukatsu T, Nikoh N, Kawai R, Koga R (2000) The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl Environ Microbiol* 66:2748–2758.
22. Baumann P, et al. (1995) Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: Intracellular symbionts of aphids. *Annu Rev Microbiol* 49:55–94.
23. Galán JE, Zhou D (2000) Striking a balance: Modulation of the actin cytoskeleton by *Salmonella*. *Proc Natl Acad Sci USA* 97:8754–8761.
24. Patel JC, Galán JE (2005) Manipulation of the host actin cytoskeleton by *Salmonella*—all in the name of entry. *Curr Opin Microbiol* 8:10–15.
25. Dale C, Plague GR, Wang B, Ochman H, Moran NA (2002) Type III secretion systems and the evolution of mutualistic endosymbiosis. *Proc Natl Acad Sci USA* 99:12397–12402.
26. Aizawa SI (2001) Bacterial flagella and type III secretion systems. *FEMS Microbiol Lett* 202:157–164.
27. Maezawa K, et al. (2006) Hundreds of flagellar basal bodies cover the cell surface of the endosymbiotic bacterium *Buchnera aphidicola* sp. strain APS. *J Bacteriol* 188: 6539–6543.