FULL PAPER *Bacteriology*

Infection of *Melissococcus plutonius* clonal complex 12 strain in European honeybee larvae is essentially confined to the digestive tract

Daisuke TAKAMATSU[1,2)]*,**, Masumi SATO[3)]** and Mikio YOSHIYAMA[4)]**

[1)]*Bacterial and Parasitic Diseases Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, 3–1–5 Kannondai, Tsukuba, Ibaraki 305–0856, Japan*
[2)]*The United Graduate School of Veterinary Sciences, Gifu University, 1–1 Yanagido, Gifu, Gifu 501–1193, Japan*
[3)]*Pathology and Pathophysiology Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, 3–1–5 Kannondai, Tsukuba, Ibaraki 305–0856, Japan*
[4)]*Honey Bee Research Unit, Animal Breeding and Reproduction Research Division, NARO Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization, 2 Ikenodai, Tsukuba, Ibaraki 305–0901, Japan*

(Received 6 July 2015/Accepted 27 July 2015/Published online in J-STAGE 8 August 2015)

ABSTRACT. *Melissococcus plutonius* is an important pathogen that causes European foulbrood (EFB) in honeybee larvae. Recently, we discovered a group of *M. plutonius* strains that are phenotypically and genetically distinct from other strains. These strains belong to clonal complex (CC) 12, as determined by multilocus sequence typing analysis, and show atypical cultural and biochemical characteristics *in vitro* compared with strains of other CCs tested. Although EFB is considered to be a purely intestinal infection according to early studies, it is unknown whether the recently found CC12 strains cause EFB by the same pathomechanism. In this study, to obtain a better understanding of EFB, we infected European honeybee (*Apis mellifera*) larvae *per os* with a well-characterized CC12 strain, DAT561, and analyzed the larvae histopathologically. Ingested DAT561 was mainly localized in the midgut lumen surrounded by the peritrophic matrix (PM) in the larvae. In badly affected larvae, the PM and midgut epithelial cells degenerated, and some bacterial cells were detected outside of the midgut. However, they did not proliferate in the deep tissues actively. By immunohistochemical analysis, the PM was stained with anti-*M. plutonius* serum in most of the DAT561-infected larvae. In some larvae, luminal surfaces of the PM were more strongly stained than the inside. These results suggest that infection of CC12 strain in honeybee larvae is essentially confined to the intestine. Moreover, our results imply the presence of *M. plutonius*-derived substances diffusing into the larval tissues in the course of infection.
KEY WORDS: clonal complex 12 strain, European foulbrood, honeybee larvae, intestinal infection, *Melissococcus plutonius*

doi: 10.1292/jvms.15-0405; *J. Vet. Med. Sci.* 78(1): 29–34, 2016

European foulbrood (EFB) is an important bacterial infectious disease of honeybee larvae. Although the causative agent, *Melissococcus plutonius*, had been thought to be a homogeneous species [4], recent multilocus sequence typing (MLST) analysis of international isolates revealed the presence of three clonal complexes (CCs) in the *M. plutonius* population, namely, CC3, CC12 and CC13 [3, 6, 10]. Among them, CC3 and CC13 include *M. plutonius* strains with fastidious cultural characteristics. They require microaerophilic to anaerobic conditions, carbon dioxide and high-potassium conditions for normal growth. Because these are the originally described characteristics of *M. plutonius* [2], strains belonging to CC3 and CC13 were referred to as typical *M. plutonius* in previous studies [1, 9, 10]. In addition to typical *M. plutonius*, we recently reported the presence of atypical *M. plutonius* strains, which are phenotypically distinct from typical *M. plutonius* [1]. These atypical strains belong to CC12 as determined by MLST [10]. Unlike *M. plutonius* strains of CC3 and CC13, strains of CC12 tested did not require high-potassium conditions for growth and can grow even under aerobic conditions on some potassium salt-supplemented media [1]. In addition, while strains of CC3 and CC13 formed a single cluster by pulsed-field gel electrophoresis (PFGE) analysis, PFGE profiles of CC12 strains clearly differed from those of CC3 and CC13 strains and formed another PFGE cluster [1], suggesting that phenotypically distinct CC12 strains have distinct genetic backgrounds.

EFB is considered to be an intestinal infection of honeybee larvae [11]. The infectious cycle of EFB begins when a larva eats brood food contaminated with *M. plutonius* [8]. According to early studies [11, 12], *M. plutonius*, which reaches the midgut of the infected larvae, is localized and starts growing between the food mass in the midgut lumen and the peritrophic matrix (or peritrophic membrane, PM) that lines the midgut epithelium. As growth continues, the bacterial mass extends towards the lumen and finally fills it almost completely. *M. plutonius* grows only in the food mass within the PM and is considered to kill its host before it, or any of the other bacteria associated with EFB, succeeds in invading the body tissues [11, 12]. However, it is unknown whether genetically diverse *M. plutonius* strains kill larvae by the same pathomechanism. Indeed, according to a large-scale epidemiological study

*CORRESPONDENCE TO: TAKAMATSU, D., Bacterial and Parasitic Diseases Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, 3–1–5 Kannondai, Tsukuba, Ibaraki 305–0856, Japan. e-mail: p1013dt@affrc.go.jp
**These authors contributed equally to this work.

conducted in England and Wales, *M. plutonius* strains from different CCs were suggested to differ in their virulence [3].

In a previous study [1], Arai *et al.* successfully reproduced EFB in artificially reared honeybee larvae by feeding them with atypical *M. plutonius* strains of CC12. The tested strains killed most of the infected larvae within 5 days, and a number of atypical *M. plutonius* were isolated from all of the larvae tested, demonstrating that CC12 strains have the ability to cause EFB in honeybee larvae. However, since CC12 strains are genetically distinct from strains of the other CCs and show peculiar phenotypic characteristics *in vitro* [1], they may have peculiar pathomechanisms, which were not observed in early studies [11, 12], for example, the strong ability to breach the PM and midgut epithelium and to invade and proliferate in the hemocoel. In this study, therefore, to investigate the pathomechanism of EFB caused by atypical *M. plutonius* of CC12, we infected European honeybee (*Apis mellifera*) larvae with a well-characterized CC12 strain and analyzed the larvae histopathologically.

MATERIALS AND METHODS

Experimental infection: Experimental infection was performed as described previously [1] with some modifications. Briefly, *M. plutonius* strain DAT561, which belongs to CC12, was cultured on KSBHI agar plates [1] at 34°C for approximately one week under anaerobic conditions, suspended in saline and adjusted to an absorbance at 600 nm of 0.1, which corresponds to approximately 3–4 × 10^7 CFU/m*l*. Then, the suspension was mixed with an equal volume of artificial diet [royal jelly (50%), sterile pure water (37%), D-glucose (6%), D-fructose (6%) and yeast extract (1%)] for preparation of the inocula. The expected final concentration of DAT561 in the inocula was approximately 1.5–2 × 10^7 CFU/m*l*.

To obtain young larvae, the queen was confined in the excluder cage in a healthy *A. mellifera* colony maintained in the apiary at the Honey Bee Research Unit, NARO Institute of Livestock and Grassland Science. On the fourth day of confinement, less than 24-hr-old worker larvae hatched in the cage were grafted onto the artificial diet diluted with an equal volume of saline in a 35-mm-diameter Petri dish and divided into control and experimental groups. The experimental groups of larvae were then fed with the artificial diet containing *M. plutonius* DAT561 prepared as described above by *ad libitum* feeding, whereas the control groups were fed with artificial diet diluted with the same volume of saline. After 24 hr of peroral infection, both groups of larvae were transferred to 24-well tissue culture plates filled with fresh artificial diet and fed with the diet by *ad libitum* feeding until a set time. In the previous experiments performed by Arai *et al.* [1], more than 90% of honeybee larvae died within 5 days by feeding them for the first 24 hr with a diet containing approximately 5 × 10^6 CFU/m*l* of DAT561; therefore, almost all larvae in the experimental groups were considered to be infected with DAT561 by the inocula used in this study. All of the larvae were incubated at 34°C in a plastic box humidified with water during the experiments.

Histopathological studies: Larvae were collected at 72 hr, 84 hr, 96 hr, 108 hr and 120 hr postinoculation (hpi) (Fig. 1) and rinsed for several sec with 70% ethanol. For routine histology by light microscopy, infected larvae were fixed in 10% phosphate-buffered formalin, dehydrated in a series of ethanol, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) reaction. Immunohistochemical (IHC) staining was performed with commercial kits [Histofine Simple Stain MAX-PO (MULTI) Nichirei and Histofine AEC Solution (for peroxidase), Nichirei, Tokyo, Japan] according to the manufacturer's instructions. A rabbit polyclonal antiserum against formalin-fixed *M. plutonius* cells generated at Nippon Biotest Laboratories Inc. (Tokyo, Japan) was diluted 8,192-fold in Dako antibody diluent with background reducing components (Dako Japan, Tokyo, Japan) and used as the primary antibody for detection of the antigen. For electron microscopy, samples were prefixed with 1% paraformaldehyde-1.25% glutaraldehyde solution, postfixed with 2% osmium tetraoxide, stained with 2% uranyl acetate, dehydrated with a graded series of ethanol and embedded in low-viscosity resin (Quetol 651 mixture, Nisshin EM, Co., Ltd., Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with an electron microscope (H-7500, Hitachi, Tokyo, Japan). Semithin sections of the samples were also submitted for the light microscopic observations after staining with methylene blue (MB) solution. In this study, a total of 30 control larvae (nos. 1–30) and 27 DAT561-infected larvae (nos. 31–57) were examined (Tables S1–S4 in the supplementary files).

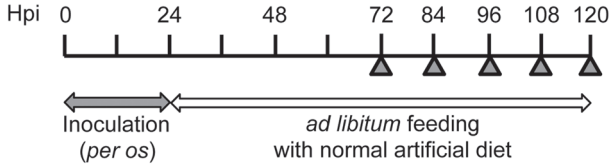

Fig. 1. Inoculation and sampling schedule for histopathological studies. Gray arrowheads indicate sampling points. Hpi, hours postinoculation.

RESULTS

Observations of non-infected control larvae: The results are summarized in Tables S1 (light microscopic observations) and S2 (electron microscopic observations) in the supplementary files.

By light microscopy, the midgut was observed in all control larvae collected until 108 hpi. However, at 120 hpi, that is, approximately six days after hatching, it was not observed clearly in all seven larvae, probably due to degeneration of the internal organs for metamorphosis. PM was observed in most of the larvae in the midguts. However, the thickness of the PM and its coverage of the midgut epithelium varied depending on the individuals. In addition, the correlation between the amounts of food in the midgut and the formation of PM on the epithelium was unclear. In nine larvae, PM covered (larva nos. 3, 5 and 8) or was considered to have covered (larva nos. 10, 17, 19, 20, 22 and 23) almost the whole surface of the

midgut epithelium and was 40–450 μm thick (Fig. 2A and 2B and Table S1). In the other larvae in which the PM was observed, the thickness sometimes reached 860 μm, but the epithelium was not fully covered by the PM (Table S1). Except for two larvae collected at 108 hpi (nos. 21 and 22; the PM might have degenerated), the surfaces of the PM were strongly positive for PAS, and the interface between the PM and lumen was clear (Fig. 2A and 2B).

By electron microscopy, PM was observed clearly only in a larva at 72 hpi (Table S2, larva no. 9). The thickness was not constant, and no food was present in the observed sections in this case. Observed PM had low electron density with a granular appearance and partially had some layers inside (Fig. 2C and 2D). However, the surface of the PM showed higher electron density than its interior, and the interface between the PM and lumen was clear, as observed by light microscopy.

Except for larvae at 120 hpi, the midgut epithelial cells were intact in all cases and had microvilli on the apical surfaces (luminal side) (Fig. 2 and Tables S1 and S2). In addition, IHC staining with anti-*M. plutonius* serum did not show any positive signals in any tissues/organs of all of the representative control larvae tested in this study (Fig. 2E). Furthermore, no bacterial cells were observed in all control larvae by both light and electron microscopies.

Light microscopic observations of strain DAT561-infected larvae: The results are summarized in Table S3 in the supplementary files. In previous studies [11, 12], it was described that "Growth of *Bacillus pluton* (a previous name of *M. plutonius*) in the infected larva begins close to the surface of the peritrophic membrane in contact with the food of the larva. As growth continues, the mass extends towards the center of the lumen of the peritrophic sac, finally filling it more or less completely". Although there were individual differences even among larvae collected at the same point, localization of the *M. plutonius* CC12 strain DAT561 within the artificially reared hosts during the course of our experimental infections was similar to those of *M. plutonius* observed in these previous studies [11, 12].

Except for 2 cases (larva nos. 47 and 51), bacterial cells were detected in the midgut lumen of all DAT561-infected larvae (Table S3). As described above, no bacteria were detected in all control larvae. In addition, the bacteria detected in the infected larvae were strongly reactive with anti-*M. plutonius* DAT561 serum. Therefore, the detected bacterial cells were considered to be the strain (*M. plutonius* DAT561) inoculated into the larvae. In most of the cases, a granular to large mass of food was also present in the midguts (Table S3). In larvae carrying a relatively small number of *M. plutonius* cells (e.g., larva no. 31), the bacterial cells in the midgut lumen were localized on the surface of the PM (Fig. 3A). As the number of *M. plutonius* cells increased, the bacterial mass of DAT561 extended towards the lumen (Fig. 3B) and filled it almost completely (Fig. 3C). In our experiments, the lumen surrounded by the PM had already been occupied by DAT561 in five of the nine larvae collected at 72 hpi (larva nos. 33 and 36–39).

Except for larva no. 50, PM was observed in all larvae in which *M. plutonius* cells were detected in the midguts. The thickness of the PM reached up to 380 μm. However, similar to non-infected control larvae, the correlation between the amounts of the midgut contents (food and bacterial cells) and the formation of the PM on the epithelium was unclear. In most of the larvae collected at 72–108 hpi, the PM covered the whole of the surface of the midgut epithelium at least in the fields of view examined, and the bacteria in the midguts were surrounded by the PM. However, in larva no. 33 collected at 72 hpi, which contained a tremendous number of *M. plutonius* cells, the PM was very thin or absent in some parts, and bacteria in the midgut directly attached to the epithelial cells (Fig. 3D). Because this larva as well as larva no. 34 was considerably smaller than the other larvae collected at 72 hpi, they were supposed to have died at an early stage. In many larvae collected at 108 hpi or later, the PM was considered to have degenerated (larva nos. 48–49 at 108 hpi and no. 57 at 120 hpi) (Fig. 3E) or covered only part of the surface of the midgut epithelium (larva nos. 52–53 and 55–57 at 120 hpi). In some of these larvae, bacteria directly attaching to the epithelial cells were observed (Fig. 3F). Interestingly, unlike non-infected control larvae, of which the PM did not show any positive signals by IHC stain (Fig. 2E), anti-*M. plutonius* serum reacted with the PM in most of the DAT561-infected larvae examined by IHC stain. In some cases, luminal surfaces of the PM were more strongly stained than the inside (Fig. 3A and 3B).

Even in DAT561-infected larvae, the midgut epithelial cells were intact and had microvilli on the apical surfaces until 96 hpi (Fig. 3A and 3G and Table S3). However, in two of the five larvae at 108 hpi (nos. 48 and 49) and three of the six larvae at 120 hpi (nos. 53, 55 and 57), the midgut epithelial cells had many vacuoles or swollen nuclei and were considered to have degenerated (Fig. 3F and 3H). In larva nos. 53, 55 and 57, microvilli on the epithelial cells were observed only partially (Table S3).

In 13 of 15 DAT561-infected larvae observed using paraffin sections, *M. plutonius* cells were observed other than in the midgut lumen (Table S3). However, most of the observed bacterial cells tended to congregate in one side of the sections, so they might be artifacts, that is, bacteria brought from the lumen through sectioning procedures. In contrast, because semi-thin sections were made from samples embedded in the hard resin, movement of bacterial cells in the sections was unlikely during sectioning procedures; therefore, the actual presence of *M. plutonius* cells located other than in the midgut lumen can be confirmed in semi-thin sections. In our study using semi-thin sections, *M. plutonius* cells were observed in the PM of six larvae (nos. 38–39 at 72 hpi and nos. 41–44 at 84 hpi), the Malpighian tubules of five larvae (no. 39 at 72 hpi, nos. 43–44 at 84 hpi, no. 45 at 96 hpi and no. 51 at 108 hpi) and hemocoel/unidentified parts of the body of four larvae (nos. 43–44 at 84 hpi and nos. 50–51 at 108 hpi) (Fig. 3I and Table S3). However, the *M. plutonius* cells located there were few in number, suggesting that the CC12 strain did not proliferate outside of the midgut lumen.

Over time, IHC-positive signals became detected at sites other than in the midgut in many larvae. In larva no. 47 at 108 hpi, although intact *M. plutonius* cells were not observed, IHC-positive signals were detected throughout the larval body (Fig. 3J). In larva no. 48 at 108 hpi, many

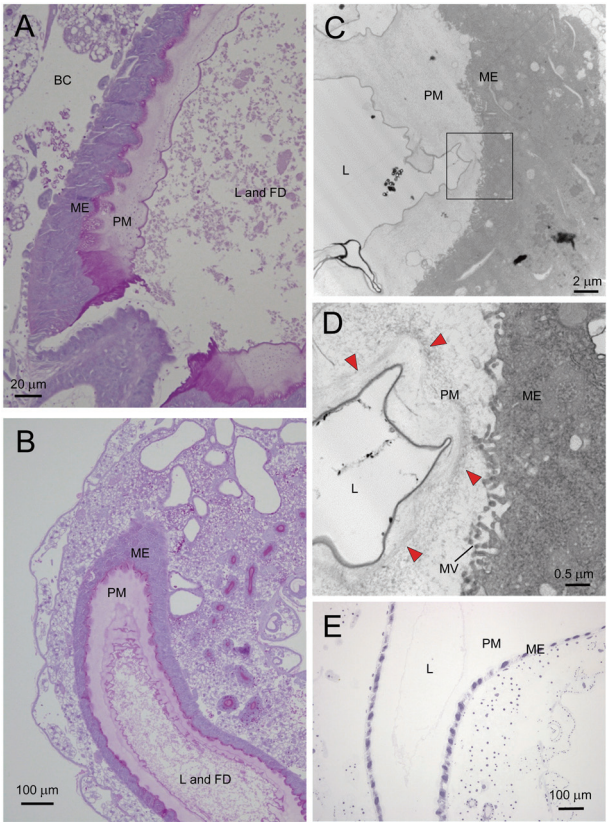

Fig. 2. Observations of non-infected control larvae. A and B, PAS staining of larva nos. 3 and 5 at 72 hpi, respectively. The surfaces of PM show strong positivity for PAS, and the interface between the PM and the lumen is clear. C, Transmission electron micrographs of larva no. 9 at 72 hpi. D, High magnification of boxed area in panel C. The PMs do not have constant thickness, having rough surfaces and low electron density with a granular appearance and partially showing some layers inside (red arrowheads in panel D). E, IHC stain of larva no. 10 at 84 hpi with anti-*M. plutonius* serum. No positive signal is observed. PM, peritrophic matrix; ME, midgut epithelial cell; L, lumen; FD, food in the midgut; BC, body cavity; MV, microvilli.

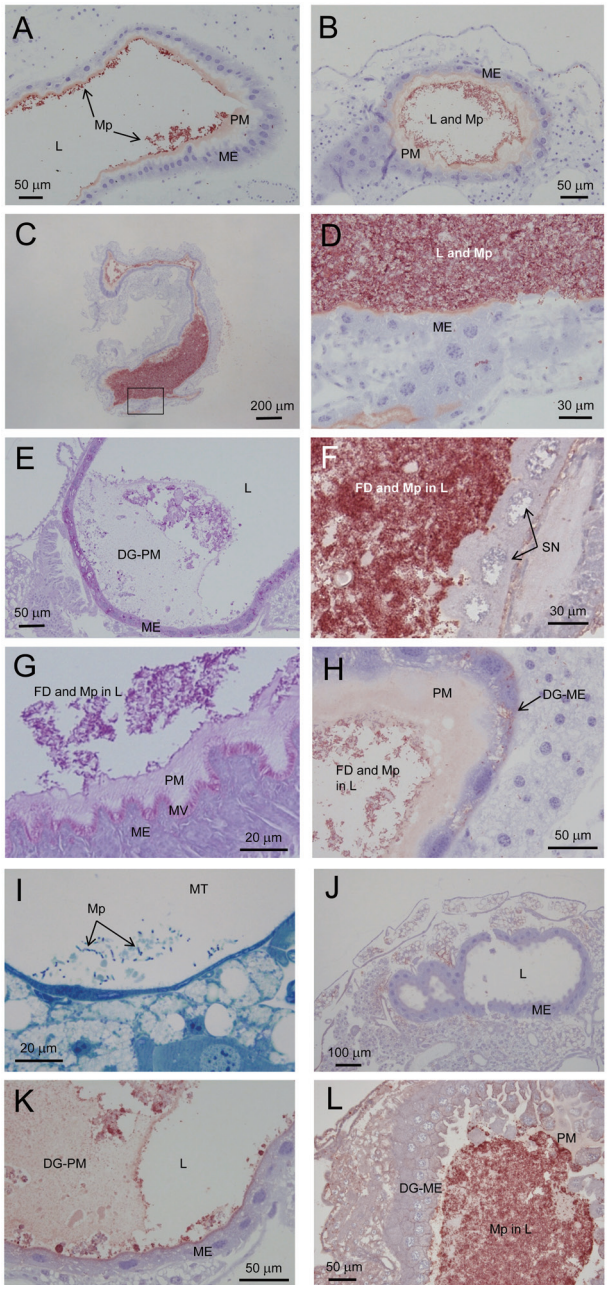

Fig. 3. Light microscopic observations of strain DAT561-infected larvae. A, IHC stain of larva no. 31 at 72 hpi. DAT561 cells are localized in the midgut lumen and attach to the surface of the PM. B, IHC stain of larva no. 32 at 72 hpi. DAT561 mass extended towards the lumen. C, IHC stain of larva no. 33 at 72 hpi. D, High magnification of boxed area in panel C. The midgut lumen is filled with DAT561 cells. E, PAS stain of larva no. 48 at 108 hpi. The PM is considered to have degenerated. F IHC stain of DAT561-infected larva no. 55 at 120 hpi. Swollen nuclei are observed in the midgut epithelial cells, and bacteria directly attach to the midgut epithelial cells. G, PAS stain of DAT561-infected larva no. 31 at 72 hpi. Intact microvilli are observed on the apical surfaces of the epithelial cells. H, IHC stain of DAT561-infected larva no. 49 at 108 hpi. The midgut epithelial cells have many vacuoles. I, MB stain of larva no. 44 at 84 hpi. A few bacteria exist in the Malpighian tubules. J, IHC stain of larva no. 47 at 108 hpi. Positive signals are detected all over the larval body. K, IHC stain of larva no. 48 at 108 hpi. Luminal side of the epithelial cells is strongly stained with the anti-*M. plutonius* serum. Degenerated PM also shows positive reaction. L, IHC stain of larva no. 53 at 120 hpi. In addition to intact bacterial cells, IHC-positive bacterial antigens are detected throughout the larval body. FD, food in the midgut; Mp, *M. plutonius*; L, lumen; PM, peritrophic matrix; DG-PM, degenerated PM; MV, microvilli; ME, midgut epithelial cell; DG-ME, degenerated ME; SN, swollen nuclei; MT, the Malpighian tubule.

IHC-positive signals were observed in the midgut epithelial cells. In particular, the luminal side of the cells was strongly reactive with the anti-*M. plutonius* serum (Fig. 3K). Some IHC-positive signals were also detected in other parts of the body in this larva. In larva nos. 53, 55 and 57 at 120 hpi, IHC-positive signals were detected throughout the larval body in addition to intact *M. plutonius* cells (Fig. 3L). Because these positive signals did not tend to congregate on one side of the sections, they were unlikely to have been artifacts produced during the sectioning procedures.

Electron microscopic observations of strain DAT561-infected larvae: The results are summarized in Table S4 in the supplementary files. *M. plutonius* cells and food mass were observed together in the midguts of all larvae, of which the lumen was included in the examined sections (Fig. 4A and Table S4). Cell division of *M. plutonius* in the lumen was prominently observed in larvae at 72–84 hpi (Fig. 4A and 4B). In some larvae collected at 84 hpi or later, many degenerated bacteria were seen in the midguts (Fig. 4C and Table S4). Apparent PM was observed only in two larvae (no. 37 at 72 hpi and no. 46 at 96 hpi) (Table S4). The PM at 72 hpi had a surface layer with high electron density, and the interface between the PM and the lumen was clear (Fig. 4D). In contrast, the PM at 96 hpi did not have such a clear interface, and it was considered to have degenerated (Fig. 4E). Some *M. plutonius* cells were observed in the degenerated PM (Fig. 4E), suggesting decreased barrier function of the PM. Except for two larvae (no. 36 at 72 hpi and no. 51 at 108 hpi), of which the midgut epithelium was not included in the examined sections, the epithelial cells had intact microvilli on the apical surfaces (Table S4), and several *M. plutonius* cells were attached to the microvilli in two of the larvae at 84 hpi (nos. 41 and 43) (Fig. 4F). *M. plutonius* cells also existed in the Malpighian tubules or some unidentified tubules with short villi in some larvae (larva no. 38 at 72 hpi, nos. 41 and 43 at 84 hpi, no. 45 at 96 hpi and no. 51 at 108 hpi) (Fig. 4G and Table S4); however, as with the light microscopic observations, the number of bacterial cells was limited.

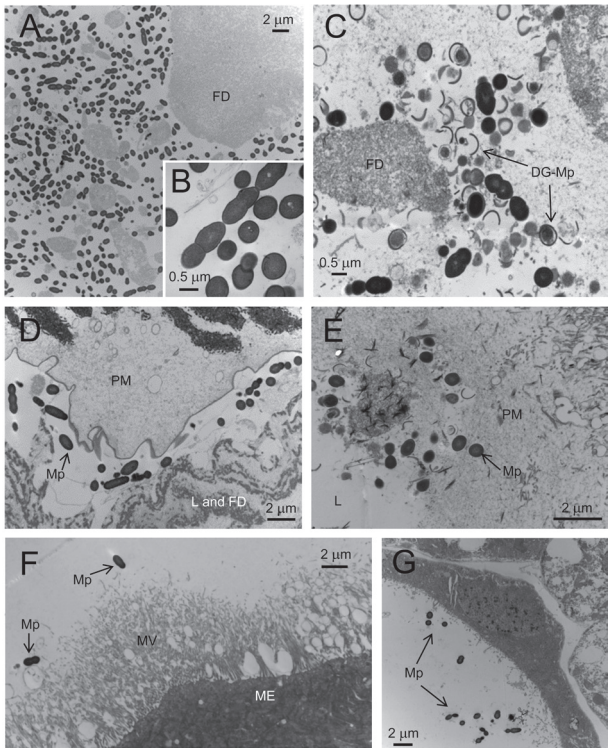

Fig. 4. Transmission electron micrographs of strain DAT561-infected larvae. A and B, *M. plutonius* cells in the midgut lumen of larva nos. 38 and 37 at 72 hpi, respectively. C, Degenerated *M. plutonius* cells in the midgut lumen of larva no. 46 at 96 hpi. D and E, Peritrophic matrix observed in larva no. 37 at 72 hpi and larva no. 46 at 96 hpi, respectively. F, Microvilli on the midgut epithelial cells and *M. plutonius* cells attaching to the microvilli observed in larva no. 41 at 84 hpi. G, *M. plutonius* cells in a tubule with short villi of larva no. 38 at 72 hpi. FD, food in the midgut; Mp, *M. plutonius*; DG-Mp, degenerated *M. plutonius*; PM, peritrophic matrix; L, lumen; MV, microvilli; ME, midgut epithelial cell.

DISCUSSION

The localization and development of *M. plutonius* within the host were described in an early study by Tarr [11] on the basis of observations using honeybee larvae of different ages and stages of EFB removed from affected bee colonies. According to this report, *M. plutonius* (described as *B. pluton* in this report) was present only between the PM and food matter, and a clear line of demarcation between *M. plutonius* and the PM was observed in 2–3-day-old larvae at the early stage of EFB. *M. plutonius* penetrated into the food mass, but not into the PM in these larvae. In a 3–4-day-old larva at a fairly advanced stage of EFB, the whole food mass had been penetrated by *M. plutonius*, but the PM was still intact. In 4–5-day-old badly affected larvae, the food mass had been entirely filled with *M. plutonius*. The PM had nearly disappeared or been disintegrated, and the intestinal epithelium had lost its characteristic structure. In a 4–5-day-old larva, which was probably dead, *M. plutonius* and secondary invaders were seen penetrating the remains of the PM in preparation for invading the body cavity of the larva. Although we cannot hope to determine at this stage which type of *M. plutonius* strains caused EFB in these larvae, on the basis of the earlier studies including this one, it is now considered that EFB is purely a disease of the digestive tract of the larva.

In this study, in spite of the atypical phenotypic characteristics of the CC12 strain *in vitro* [1], the course of EFB caused by strain DAT561 was similar to those observed in previous studies [11, 12]. DAT561 cells were located on the surface of the PM at the early stage of infection. They multiplied vigorously in the midguts and eventually occupied the midgut lumen almost completely. In badly affected larvae, the PM and midgut epithelial cells had degenerated. In our study, *M. plutonius* was also detected in organs/tissues other than the midgut lumen by light microscopy using semithin sections as well as by electron microscopy; however, compared with the etiological agent of American foulbrood (*Paenibacillus larvae*) that breaches the gut epithelium and invades the hemocoel in larvae [13], the number of *M.*

plutonius cells located outside the gut lumen was small in most cases. In one case (larva no. 53), a certain number of *M. plutonius* cells were observed throughout the larval body (Table S3). However, since the larval tissues including the midgut epithelium were disintegrated in this larva, the bacteria may have invaded the body cavity after the death of the larva in this case. In addition, because this larva was examined in paraffin sections, some of the observed bacterial cells might have been artifacts, as indicated in the Results. In some larvae, a small number of *M. plutonius* cells were also observed in the Malpighian tubules (Table S3). Malpighian tubules are the organs that play a role analogous to the kidney of vertebrate animals. Because the tubules connect the digestive tract at the midgut-hindgut junction, *M. plutonius* observed in the Malpighian tubules may be cells that leaked out from the midgut. Therefore, our observations strongly suggest that the infection of CC12 strains in honeybee larvae is also essentially confined to the digestive tract, and the bacteria do not invade or proliferate in the body cavity actively.

IHC analyses of *M. plutonius*-infected larvae, which were carried out for the first time in EFB studies, detected positive signals in the larval bodies in addition to intact bacterial cells. Such signals were observed throughout the larval body in some badly affected larvae at 108 hpi or later (Fig. 3J–3L and Table S3, larva nos. 47, 48, 53, 55 and 57). However, the larval tissues including the midgut epithelium had degenerated in these larvae. In addition, disintegrated *M. plutonius* cells were observed in the midguts at 84 hpi or later (Fig. 4C and Table S4). Therefore, the signals detected in the deep tissues of badly affected larvae might be debris of *M. plutonius* cells infiltrated into the larval body after the death of the hosts. In contrast, IHC-positive signals in the PM were already detected in many larvae at 72 hpi, and disintegrated *M. plutonius* cells were hardly detected at this point (Table S3). Because no IHC-positive signals were detected in all control larvae tested, these results imply that some molecules secreted or released from *M. plutonius* cells infiltrated into the PM at the early stage of infection. The PM is a chitin and glycoprotein layer that lines the midgut of most insect species. It contributes to efficient nutrient acquisition and recycling of digestive enzymes by compartmentalizing digestive processes. The PM also protects the midgut epithelium from mechanical and biochemical damage by abrasive food particles, digestive enzymes and pathogens [7]. In the development of American foulbrood, degradation of the PM occurs in the gut of *P. larvae*-infected larvae, which enables *P. larvae* to access the underlying epithelial cells directly and attack them [5]. At the advanced stage of EFB, the PM also tended to have degenerated or be absent in some parts. However, it is unknown at present whether molecules that reacted with anti-*M. plutonius* serum have any effects on the integrity of the PM, that is, whether they are virulence factors of *M. plutonius*. It is also unknown whether similar IHC-positive signals appear when larvae are infected with *M. plutonius* strains of other CCs. Therefore, to obtain a more comprehensive understanding of this disease, further studies including the identification of virulence factors and investigation of the distribution of these factors in the *M. plutonius* population will be needed.

ACKNOWLEDGMENTS. This work was supported by JSPS KAKENHI Grant Number 25292200. We thank M. Kobayashi and M. Shimada for technical assistance in the histopathological analysis. We are also grateful to M. Okura for technical assistance in the figure preparation.

REFERENCES

1. Arai, R., Tominaga, K., Wu, M., Okura, M., Ito, K., Okamura, N., Onishi, H., Osaki, M., Sugimura, Y., Yoshiyama, M. and Takamatsu, D. 2012. Diversity of *Melissococcus plutonius* from honeybee larvae in Japan and experimental reproduction of European foulbrood with cultured atypical isolates. *PLoS ONE* **7**: e33708. [Medline] [CrossRef]
2. Bailey, L. and Collins, M. D. 1982. Reclassification of '*Streptococcus pluton*' (White) in a new genus *Melissococcus*, as *Melissococcus pluton* nom. rev.; comb. nov. *J. Appl. Bacteriol.* **53**: 215–217. [CrossRef]
3. Budge, G. E., Shirley, M. D. F., Jones, B., Quill, E., Tomkies, V., Feil, E. J., Brown, M. A. and Haynes, E. G. 2014. Molecular epidemiology and population structure of the honey bee brood pathogen *Melissococcus plutonius*. *ISME J.* **8**: 1588–1597. [Medline] [CrossRef]
4. Forsgren, E. 2010. European foulbrood in honey bees. *J. Invertebr. Pathol.* **103**: S5–S9. [Medline] [CrossRef]
5. Garcia-Gonzalez, E. and Genersch, E. 2013. Honey bee larval peritrophic matrix degradation during infection with *Paenibacillus larvae*, the aetiological agent of American foulbrood of honey bees, is a key step in pathogenesis. *Environ. Microbiol.* **15**: 2894–2901. [Medline]
6. Haynes, E., Helgason, T., Young, J. P. W., Thwaites, R. and Budge, G. E. 2013. A typing scheme for the honeybee pathogen *Melissococcus plutonius* allows detection of disease transmission events and a study of the distribution of variants. *Environ. Microbiol. Rep.* **5**: 525–529. [Medline] [CrossRef]
7. Hegedus, D., Erlandson, M., Gillott, C. and Toprak, U. 2009. New insights into peritrophic matrix synthesis, architecture, and function. *Annu. Rev. Entomol.* **54**: 285–302. [Medline] [CrossRef]
8. Shimanuki, H. 1997. Bacteria. pp. 33–56. *In*: Honey Bee Pests, Predators, and Diseases, 3rd ed. (Morse, R.A. and Flottum, K. eds.), The A. I. Root Company, Medina.
9. Takamatsu, D., Arai, R., Miyoshi-Akiyama, T., Okumura, K., Okura, M., Kirikae, T., Kojima, A. and Osaki, M. 2013. Identification of mutations involved in the requirement of potassium for growth of typical *Melissococcus plutonius* strains. *Appl. Environ. Microbiol.* **79**: 3882–3886. [Medline] [CrossRef]
10. Takamatsu, D., Morinishi, K., Arai, R., Sakamoto, A., Okura, M. and Osaki, M. 2014. Typing of *Melissococcus plutonius* isolated from European and Japanese honeybees suggests spread of sequence types across borders and between different *Apis* species. *Vet. Microbiol.* **171**: 221–226. [Medline] [CrossRef]
11. Tarr, H. L. A. 1938. Studies on European foul brood of bees. IV. On the attempted cultivation of *Bacillus pluton*, the susceptibility of individual larvae to inoculation with this organism and its localization within its host. *Ann. Appl. Biol.* **25**: 815–821. [CrossRef]
12. White, G. F. 1920. European foul brood. *Bull. U.S. Dep. Agric.* no.810.
13. Yue, D., Nordhoff, M., Wieler, L. H. and Genersch, E. 2008. Fluorescence *in situ* hybridization (FISH) analysis of the interactions between honeybee larvae and *Paenibacillus larvae*, the causative agent of American foulbrood of honeybees (*Apis mellifera*). *Environ. Microbiol.* **10**: 1612–1620. [Medline] [CrossRef]