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Cell Structure of the Infectious Form of Holospora, an Endonuclear Symbiotic Bacterium of the Ciliate Paramecium

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ABSTRACT—Under a phase-contrast microscope, the infectious form of Holospora obtusa appears to consist of three regions in tandem: a dark region, a refractile region, and a small dark tip in the refractile region. By electron microscopy, this bacterium appears to consist of a cytoplasmic region, a periplasmic region, and a small electron-translucent tip in the periplasmic region. It had previously been thought that these three regions in each microscopy respectively corresponded to one another. However, indirect immunofluorescence microscopy with monoclonal antibodies, IF-3-1 and IF-3-2, specific for periplasmic proteins of H. obtusa and an antiserum raised against a cytoplasmic stress protein, the GroEL homologue of aphid endosymbiotic bacterium, showed that the dark region was the periplasm and the refractile region was the cytoplasm. This shows that the small dark tip does not correspond to the small electron-translucent tip, and that the electrontranslucent tip should be present at the opposite end. We found that this special tip could be identified with Nomarski- and phase-contrast optics at the end of the dark region when the infectious form was embedded in 30% gelatin. The same correspondences between the light and the electron microscopic structures were also confirmed in the infectious form of the micronucleus-specific bacterium H. recta.

INTRODUCTION

Species of the Gram-negative bacterium Holospora are endonuclear symbionts of species of the ciliate Paramecium. So far, nine Holospora species have been described (Fokin et al., 1996). All species show species-specificity and nucleusspecificity in their habitat. For example, H. obtusa infects and grows in the macronucleus of P. caudatum, while H. elegans, H. undulata and H. recta infect and grow in the micronucleus of P. caudatum. These bacteria show two distinct forms in their life cycle: an infectious long form and a reproductive short form. The infectious form of Holospora species consists of three regions in tandem under a phase-contrast microscope: a dark region, a refractile region, and a small dark tip in the refractile region (Fujishima and Hoshide, 1988). Under a transmission electron microscope, the cytoplasmic region occupies only about half the length of the infectious form enclosed by the periplasmic layer, and the remaining region of the infectious form is occupied by a large periplasmic region with a small electron-translucent tip (Fujishima and Hoshide, 1988). Namely, the infectious form consists of three regions in tandem: a cytoplasmic region, a periplasmic region, and a small

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electron-translucent tip in the periplasmic region. The infectious form always invades the host nucleus with this small electron-translucent tip first (Fujishima and Fujita, 1985). Although the mechanism of nucleus-specific infection of Holospora species has not yet been clarified, it is at least clear that they possess an ability to recognize some differences between two kinds of host nuclei and that they invade the specific nucleus by this special tip. Therefore, this tip is called a "recognition tip".

Based on the assumption that the small dark tip under a phase-contrast microscope corresponds to the small electrontranslucent tip, it had been conjectured that the dark region under a phase-contrast microscope should correspond to the cytoplasmic region and the refractile region corresponds to the periplasmic region (Görtz and Dieckmann, 1980; Fujishima and Hoshide, 1988). However, unexpectedly, fluorescence of DNA-specific fluorochrome, 4', 6-diamidino-2-phenylindole (DAPI) appeared only in the refractile region of the infectious form of H. obtusa (Fujishima and Hoshide, 1988). Furthermore, a narrow cytoplasmic extrusion in the central part of the periplasmic region was found in H. obtusa (Görtz et al., 1989). Therefore, it was suspected that localization of DNA in this narrow cytoplasmic extrusion made the refractile region DAPIpositive (Dohra et al., 1994). However, Dohra and Fujishima (1992) showed that a monoclonal antibody, which had been

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confirmed to react with a periplasmic protein of 15 kDa of H. obtusa by immunoelectron microscopy (Dohra et al., 1994; Fujishima et al., 1997), had labeled only the DAPI-negative region of the infectious form. This result suggests the other possibility that the dark region corresponds to the periplasmic region and the refractile region corresponds to the cytoplasmic region. In order to test this hypothesis, in this study, we reexamined the exact correspondences between the light and the electron microscopic structures of the infectious form of H. obtusa using two kinds of monoclonal antibodies, IF-3-1 and IF-3-2, specific for periplasmic proteins of the infectious form (Dohra et al., 1994; Fujishima et al., 1997), and an antiserum specific for GroEL homologue in the cytoplasm (Dohra et al., 1998). Furthermore, we established a new method which made it possible to observe the recognition tip of H. obtusa with Nomarski- and phase-contrast optics. In order to determine whether the correspondences between the light and the electron microscopic cell structures in H. obtusa were a common feature in other Holospora species, we also examined the morphology of the infectious form of the micronucleusspecific H. recta.

MATERIALS AND METHODS

Paramecium cells and culture conditions

The *H. obtusa*-bearing strain RB-1 (mating type E, syngen unknown) and the *H. recta*-bearing strain P20-16 of *P. caudatum* (mating type and syngen unknown) were cultivated in modified lettuce juice medium at 25°C, as described previously (Fujishima *et al.*, 1991), except that KH₂PO₄ was used instead of NaH₂PO₄ • 2H₂O in the medium.

Antibodies

Monoclonal antibodies, IF-3-1 and IF-3-2, which are specific for the 39 kDa and 15 kDa periplasmic proteins, respectively, of the infectious forms of *H. obtusa* (Dohra *et al.*, 1994), were used. A rabbit antiserum raised against a purified GroEL homologue of an aphid symbiont, was kindly provided by Dr. Hajime Ishikawa, Graduate School of Science, University of Tokyo.

Isolation of infectious form of H. obtusa

Infectious long forms of *H. obtusa* and *H. recta* were isolated as described previously (Fujishima *et al.*, 1990a, b; Kawai and Fujishima, 1996). Briefly, a *Paramecium* cell pellet was suspended in an equal volume of 0.25 M sucrose and homogenized with a Teflon homogenizer on ice. The homogenates were then centrifuged in 70% (v/v) Percoll at 39,000 × g for 30 min at 4°C. For *H. obtusa* and *H. recta*, bands of bacteria with buoyant densities of 1.16 g/ml and 1.13-1.16 g/ml, respectively, were harvested and washed twice with 10 mM Na, K-phosphate buffer, pH 6.5 (NaKP), by centrifuging at 2,000 × g for 10 min at 4°C.

Light microscopy and indirect immunofluorescence microscopy

The infectious forms suspended in NaKP, were dried on glass slides, and stained with 1 μ g/ml of 4', 6-diamidino-2-phenylindole (DAPI) dissolved with phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄ • 12H₂O, 1.47 mM KH₂PO₄, pH 7.2, for 10 min at room temperature. For observation of the recognition tip of the infectious form under Nomarski-differential-interference-contrast and phase-contrast optics, the slides were washed 3 times with NaKP for 5 min each, mounted in NaKP containing 30% (w/v) gelatin, and then observed.

In the case of *H. recta*, the infectious forms suspended in NaKP were fixed with 4% (w/v) paraformaldehyde dissolved with PBS. The cells were then washed twice with PBS for 5 min each by centrifuging at 2,000 × g at 4°C, suspended with 1 µg/ml of DAPI dissolved with PBS, and then observed.

Indirect immunofluorescence microscopy was carried out as follows. Isolated infectious forms of *H. obtusa* suspended in NaKP, were dried on glass slides, fixed in an ethanol/acetic acid (3 : 1) mixture for 10 min at room temperature, washed with PBS for 15 min and incubated with the primary antibody for 30 min at room temperature. The cells were then washed with PBS for 15 min at 4°C, and incubated with FITC-conjugated secondary antibody for 30 min at room temperature. Then, the cells were washed twice with PBS for 15 min each at 4°C, stained with 1 μ g/ml of DAPI dissolved with PBS for 10 min at room temperature, and observed with a fluorescence microscope (Olympus, BH2-RFL).

Transmission electron microscopy

H. recta-bearing paramecia in the early stationary phase of growth were fixed in 0.05 M Na, K-phosphate buffer, pH 7.2, containing 2.5% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde for 1 h at room temperature. The cells were then washed 3 times with the same buffer for 20 min and washed once with deionized water for 20 min at room temperature. The cells were dehydrated in an ethanol concentration series of 30-100% (v/v), and then embedded in LR white resin. Thin sections on nickel grids were stained with uranyl acetate for 10-15 min, washed with deionized water and observed with a transmission electron microscope (JEOL JEM-1200EX) at 80 kV.

RESULTS

Discrimination of a periplasmic and a cytoplasmic region of the infectious form of *H. obtusa* by indirect immunofluorescence microscopy

The infectious form of *H. obtusa* consists of three regions in tandem under a phase-contrast microscope: a dark region, a refractile region that has two DAPI-positive nucleoids (Fujishima and Hoshide, 1988; Fujishima et al., 1990; Fujishima et al., 1991), and a small dark tip in the refractile region. Indirect immunofluorescence microscopy showed that IF-3-1 and IF-3-2 antibodies specific for periplasmic proteins labeled only the DAPI-negative region of the infectious form of H. obtusa (Fig. 1). In contrast, an antiserum specific for a cytoplasmic GroEL homologue reacted with the other half of the bacterium, where DAPI-positive nucleoids were present. Fig. 1 indicates that the periplasmic region is the dark region, and the cytoplasmic region is the refractile region. This result contradicts our previous assumption that DAPI fluorescence at the periplasmic region should be due to the localization of DNA in a narrow cytoplasmic extrusion in the central part of the periplasmic region (Dohra et al., 1994). Namely, the present observation shows that an electron-translucent tip (the recognition tip) is not the small dark tip at the end of the refractile region, because it is known that the recognition tip is present at the end of the periplasmic region of the infectious form by electron microscopy (Schmidt et al., 1987; Fujishima and Hoshide, 1988; Görtz et al., 1989; Görtz et al. 1990; Wiemann and Görtz, 1991; Dohra et al., 1994; Fujishima et al., 1997).

Cell Structure of Holospora



Fig. 1. Fluorescence micrographs of the infectious form of *H. obtusa*. Infectious forms were labeled with a periplasm-specific IF-3-1 monoclonal antibody (A), a periplasm-specific IF-3-2 monoclonal antibody (B), and a cytoplasm-specific GroEL antiserum (C). FITC-fluorescence (A, B and C). DAPI fluorescence (D, E and F). A and D, B and E, and C and F show the same cell. Bar: 5 μm.

Visualization of a recognition tip of the infectious form of *H. obtusa*

If the dark and the refractile regions under a phase-contrast microscope correspond to the periplasmic and the cytoplasmic regions, respectively, the recognition tip should appear at the end of the DAPI-negative periplasmic region. However, the recognition tip-like structure could not be distinguished at the end of the dark region under the phase-contrast microscope. We found that a pale tip which seemed to be the recognition tip could be detected at one end of the



Fig. 2. Visualization of a recognition tip under Nomarski-differential-interference-contrast and phase-contrast microscopes. Cells were suspended in 30% gelatin. (A) Nomarski-differential-interference contrast micrograph; (B) phase-contrast micrograph; (C) DAPI fluorescence micrograph. A, B and C show the same cell. Arrows show the boundary between the periplasmic region (left) and the cytoplasmic region (right) with two DAPI-positive nucleoids. Arrowheads show the recognition tip. Bar: 5 μ m.

bacterium under both Nomarski- and phase-contrast optics (Fig. 2A and B), when the infectious forms were embedded in 30% (w/v) gelatin which had been originally developed for observation of nucleoids in *Bacillus* (Mason and Powelson, 1956). Judging from the position of two DAPI-positive spots in the same specimen (Fig. 2C), this pale tip apparently corresponds to the recognition tip at the end of the DAPI-negative periplasmic region. The cytoplasmic region bearing the two DAPI-positive spots could be detected by phase-contrast microscopy, because it was fairly refractile (Fig. 2B) and the other half of the region which corresponded to the periplasmic region looked dark except for the pale tip. After gelatin treatment, the small dark tip that was detected at the end of the refractile region in phase-contrast disappeared (Fig. 2B).

Thus, this observation shows that the dark and the refractile regions under a phase-contrast microscope correspond to the periplasmic and the cytoplasmic regions, respectively. In other words, the small dark tip at the end of the refractile region is not the recognition tip.

Discrimination of a cytoplasmic region, a periplasmic region and a recognition tip in the infectious form of *H. recta*

Unlike the infectious forms of H. obtusa (Fujishima and Hoshide, 1988; Fujishima et al., 1990; Fujishima et al., 1991) and H. elegans (Görtz and Dieckmann, 1980), the infectious form of H. recta consisted of three regions under a phasecontrast microscope; a refractile region and a dark region with a small dark tip (Fig. 3). Compared with H. obtusa (Fujishima and Hoshide 1988) and H. elegans (Görtz and Dieckmann, 1980), H. recta has a short length and its refractile region and the dark region are not as clear. However, the small dark tip of the end of the dark region is larger than the small dark tip of H. obtusa in Fig. 2 and is easily observable under phase- and Nomarski-microscopes without embedding the bacterium in gelatin (Fig. 3A and B). As shown in Fig. 3A and B, the infectious form of H. recta slightly curved, and has a rounded end and a tapered end. Therefore, unlike H. obtusa and H. elegans, both ends are easily distinguishable by their morphologies. Namely, the small dark tip is present at the rounded end adjacent to the dark region, and the tapered end is the end of the



Fig. 3. Morphology of the infectious form of *H. recta.* (A) Nomarskidifferential-interference contrast micrograph; (B) phase-contrast micrograph; (C) DAPI fluorescence micrograph. Arrows show the boundary between the dark region (left) and the refractile region (right) bearing the DAPI-positive area. The dark tip in B (arrowhead) is also visible in A (arrowhead). Bar: 5 μ m.

refractile region (Fig. 3A and B). As shown in Fig. 3C, DAPIfluorescence appeared at the refractile region.

Fig. 4 shows the ultrastructure of the infectious form of *H.* recta in the host micronucleus. An electron-translucent tip (recognition tip) was present at the rounded end adjacent to the periplasmic region, indicating that this tip is the dark tip under a phase-contrast microscope. The length of the recognition tip is about 1 μ m and its maximum diameter is about 0.6 μ m. On the other hand, the cytoplasmic region had a tapered end.

Thus, it is concluded that the small dark tip, the dark region and the refractile region with a tapered end observed in the light microscopic structures correspond to the recognition tip, the periplasmic region and the cytoplasmic region, respectively.

DISCUSSION

In the present study, we found that the recognition tip became distinguishable in both Nomarski- and phase-contrast optics when the infectious form of H. obtusa was embedded in 30% gelatin. In gelatin-embedded specimens, although nucleoids could not be distinguished, the dark and the DAPIpositive refractile region were still recognized. Previously we showed with a scanning electron microscope that one end of the infectious form of *H. obtusa* had a rough surface that was easily depressed by surface tension (Fujishima and Hoshide, 1988; Fujishima et al., 1990b). Therefore, such a different nature of the recognition tip may be responsible for the appearance of the tip in 30% gelatin. Wiemann and Görtz (1991) reported that the recognition tip-like structure could be detected with phase-contrast when the infectious form of H. obtusa was extracted with chloroform. However, the difference between the dark and the refractile regions of the bacterium was obscure in this preparation.

This is the first report on an exact correspondence between the light and the electron microscopic structures. Namely, the dark region of the infectious form in phase-contrast was the periplasmic region and the refractile regions was the cytoplasmic regions (Fig. 5). These correspondences were also the same in the micronucleus-specific H. recta. However, unlike the dark regions in the infectious forms of H. obtusa and H. recta, the dark region in the infectious form of the micronucleus-specific H. elegans was DAPI-positive (Görtz and Dieckmann, 1980). The infectious form of H. elegans consisted of the dark and the refractile region with the dark small tip in phase contrast, and ultrastructurally it consisted of the recognition tip, the periplasmic and the cytoplasmic region, as was the case with H. obtusa and H. recta. We cannot eliminate the possibilities that the dark region in H. elegans is the cytoplasmic region or that nucleoids are present in a narrow cytoplasmic extrusion in the periplasmic region even though neither of these features occur in H. obtusa and H. recta.



Fig. 4. Ultrastructures of the infectious forms of *H. recta* in the host micronucleus. The arrowhead shows an electron-translucent recognition tip. The arrow shows the boundary between an electron-dense periplasmic region (left of arrow) and a cytoplasmic region (right of arrow). Note that the recognition tip is present at the rounded end adjacent to the periplasmic region, and that the cytoplasmic region has a tapered end. Bar: 1 µm.



Recognition tip (homogeniously electron-translucent tip)

Fig. 5. Schematic representation of morphological characters of the infectious forms of H. obtusa. A, phase-contrast. The infectious form appears to consist of three regions in tandem: a dark region, a refractile region, and a small dark tip. B, DAPI-fluorescence. Two white spots show DAPI-positive nucleoids. C, indirect immunofluorescence labeled with a cytoplasm-specific GroEL antiserum. D, indirect immunofluorescence labeled with a periplasm-specific monoclonal antibody raised against 39 kDa or 15 kDa protein of H. obtusa. White regions in C and D show FITC-fluorescence positive regions. E, Nomarskidifferential-interference contrast in 30% gelatin. F, transmission electron microscopy. Noted that both the refractile region and the small dark tip in A belonged to a cytoplasmic region as revealed in C, and that majority of the dark region in A was a periplasmic region as revealed in D. Namely, a small dark tip in A does not correspond to a recognition tip. The recognition tip was identified when the bacterium was embedded in 30% gelatin in H. obtusa, as shown in E.

However, the correspondences of the light microscopic morphologies and the DAPI-positive region should be examined again in *H. elegans* in the future.

Under a phase-contrast microscope, the infectious form of *Holospora* species has a small dark tip in the refractile region. However, ultrastructurally, no special structures are present at the end of the cytoplasmic region (Fujishima and Hoshide, 1988; Fujishima *et al.*, 1990b; Wiemann and Görtz, 1991). Because the periplasmic layer appears a little thick at the end of the cytoplasmic region in a photograph of Wiemann and Görtz (1991), this may be a cause for the small dark tip in the refractile region.

In *H. recta*, DAPI-fluorescence appeared throughout the refractile region (Fig. 3C). This seems to be an artifact of cell preparation. When the cells were dried on a glass slide prior to DAPI-staining, the fluorescence appeared as clusters in the cytoplasm near the border between the cytoplasmic and periplasmic regions (Kawai and Fujishima, 1996). However, the air-drying method made the three regions indistinct in phase-contrast, so that we did not use this method in this study.

The two DAPI-fluorescence spots in *H. obtusa* show the presence of nucleoids. However, no nucleoidal structures have yet been observed in the cytoplasm by electron microscopy, and no nucleoid-specific antibody has been developed in *H. obtusa.* Also, no recognition tip-specific antibody has yet been developed for any *Holospora* species, even though the substances at the surface of this tip and in the electron-translucent materials in the tip are expected to be responsible for distinguishing two kinds of host nuclei and for penetration of the host nuclear envelope by the bacterium.

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