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Partial Characterization and Cryopreservation of the Isolated Symbiotic Bacterium *Holospora recta* of the Ciliate *Paramecium caudatum*

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ABSTRACT—*Holospora recta* is a micronucleus-specific symbiotic bacterium of the ciliate *Paramecium caudatum*. This bacterium cannot grow outside the host cell. We isolated the infectious form of this bacterium from the host homogenates by 70% Percoll density gradient centrifugation. DNA in the infectious form of *H. recta* appeared as a large cluster in the cytoplasmic region near the large periplasm. This was not observed with the infectious form of a macronucleus-specific symbiont of *P. caudatum*, *H. obtusa*. The isolated infectious form could infect the micronucleus of *P. caudatum* and differentiated into the reproductive form within 18 hr after the infection at 25°C. However, not only was this bacterium unable to infect the micronucleus of *P. bursaria*, but it was also unable to infect the micronucleus of *P. multimicronucleatum* or *P. novaurelia*, even though the latter two species are morphologically closely related to *P. caudatum*. We succeeded in the cryopreservation of this bacterium, as cells stored at -85°C for 127 days maintained their infectivity and reproducibility.

INTRODUCTION

Holospora species are Gram-negative symbiotic bacteria of ciliates of the genus *Paramecium*. All of the nine *Holospora* species that have been described (Borchsenius *et al.*, 1983; Fokin, 1989, 1991; Fokin and Sabaneyeva, 1993; Hafkine, 1890; Ossipov *et al.*, 1975, 1980; Preer, 1969) show species-specificity and nucleus-specificity in their habitats. For example, *H. obtusa* is the macronucleus-specific, but *H. recta*, *H. elegans* and *H. undulata* are the micronucleus-specific bacteria of *P. caudatum* (Hafkine, 1890). *H. caryophila* is a macronucleus-specific bacterium of both *P. biaurelia* (Preer, 1969) and *P. novaurelia* (Fokin *et al.*, 1996). *H. acuminata* (Ossipov *et al.*, 1980) is a micronucleus-specific and *H. curviuscula* (Borchsenius *et al.*, 1983) is a macronucleus-specific bacterium of *P. bursaria*. *H. bacillata* (Fokin, 1989; Fokin and Sabaneyeva, 1993) is a macronucleus-specific of *P. woodruffi* and *P. calkinsi*, and *H. curvata* (Fokin and Sabaneyeva, 1993) is a macronucleus-specific bacterium of *P. calkinsi*. Although they can distinguish *Paramecium* species and two different nuclei, control mechanisms of species-specific and nucleus-specific infection is not known. *H. obtusa* infects the macronucleus of *H. elegans*-bearing cell and *H. elegans* also infects the micronucleus of *H. obtusa*-bearing cell, but always a former occupant can remain in the host cell (Fujishima and Görtz, unpublished data), notwithstanding that each *Holospora* is present in different nuclei in the cell. On the other hand, *H. elegans*-bearing cell could not produce their offspring even if the bacteria were removed by treatment with antibiotics (Görtz and Fujishima, 1983), and usually *Holospora*-bearer showed decreased fission ratio, and paramecia do not need holosporas for their growth. Therefore, *Holospora* species are considered as parasitic bacteria. However, regardless of such harmful effects of the holosporas, they are easily found in paramecia living in relatively cold area such as Northern Europe and the Kamchatka peninsula. Therefore, their existence may give some merits for their host survival in cold temperature.

Holospora species have two distinct forms in their life cycle: a reproductive short form and an infectious long form. When the host cell grows, the reproductive form also grows by binary fission in the host nucleus. When the host ceases binary fission as a result of starvation, the reproductive form ceases binary fission and differentiates into the infectious form. The infectious form is unable to grow by binary fission in the host nucleus, but is able to escape from the host cell (Wiemann, 1989) invades the cytoplasm of a new host through the host digestive vacuole and then enter the target nucleus with its special tip (Görtz and Wiemann, 1989).

Because *Holospora* species cannot grow outside the host cell, they have to be isolated from mass-cultured host cells when large numbers of cells are needed for biochemical experiments. Furthermore, although infection can be induced by mixing *Holospora*-free paramecia with *Holospora*-bearing *Paramecium* homogenates (Fokin and Sabaneyeva, 1993; Fujishima and Fujita, 1985; Görtz and Dieckmann, 1980;

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Ossipov *et al.*, 1975, 1993), reproducible results with respect to the timing of the infection and infection ratio are difficult to obtain because of the harmful effect of *Paramecium* debris in the homogenates. Therefore, isolated infectious forms instead of the host homogenates are needed, especially for studies on early events in the infection process and on recognition mechanism of two kinds of the host nuclei by *Holospora* species.

Holosporas were first isolated from host homogenates by Preer (1969), who used an Ecteola column to isolate the macronucleus-specific bacterium *H. caryophila* from *P. biaurelia*. Later, *H. obtusa* has been isolated by Fujishima and Nagahara (1985) and *H. elegans* has been isolated by Schmidt *et al.* (1987), using Percoll density gradient centrifugation.

In the present study, we attempted to isolate the infectious form of *H. recta* in a way that maintained its infectivity, and to compare it with *H. obtusa* with respect to its morphology and timing of differentiation from the infectious form to the reproductive form. We also examined whether the infectious form of *H. recta* can infect other *Paramecium* species that are closely related to *P. caudatum*, as previously shown with *H. obtusa* (Fujishima and Fujita, 1985). Furthermore, in order to maintain this symbiotic bacterium in the laboratory without culturing, we attempted cryopreservation of the isolated infectious form of *H. recta*.

MATERIALS AND METHODS

Cell and culture conditions

P20-16, a *Holospora recta*-bearing strain of *P. caudatum* (mating type and syngen unknown) and RB-1, a strain of *P. caudatum* that does not have *H. recta* (mating type E, syngen unknown) were cultivated in modified lettuce juice medium at 25°C, as described in our previous paper (Fujishima *et al.*, 1990a) except that KH_2PO_4 was used instead of $NaH_2PO_4 \cdot 2H_2O$ in the medium. Other *Paramecium* strains that were used included the *P. multimicronucleatum* strain 49B (syngen 2, mating type IV) and strain 53B (syngen 2, mating type III), the *H. caryophila*-bearing *P. novaurelia* strain 91YB1-3 and the *H. curviuscula*-bearing *P. bursaria* strain ET50-16. These strains were kindly provided by Dr. D. V. Ossipov, Dr. I. I. Skoblo and Dr. M. Rautian, St. Petersburg State University, Russia.

Isolation of H. recta

Isolation of the infectious form of *H. recta* was carried out as described previously (Fujishima *et al.*, 1990a, b) with a slight modification. Briefly, 12 liters of medium containing *H. recta*-bearing paramecia in the stationary phase of growth were harvested by flow-through centrifugation at 700 × g for 30 min. The cells were then washed twice with Dryl's solution (Dryl, 1959) by centrifuging at 800 × g for 3 min at 4°C, washed once with 0.25 M sucrose by the same centrifugation and homogenized in a Teflon homogenizer. The homogenates were centrifuged in 70% (v/v) Percoll (Pharmacia LKB Biotechnology) at 37,500 × g (instead of 38,900 × g) for 30 min at 4°C. A bacterial band denser than 1.13 g/ml was collected and washed twice with 10 mM Na, K-Phosphate buffer (Na, K-PB), pH 6.5, by centrifuging at 2,000 × g for 10 min at 4°C. Densities were visualized with a density marker beads kit (Pharmacia LKB Biotechnology).

Infection

Isolated cells of the infectious form were mixed with *Paramecium* cells at densities of about 1×10^5 bacteria/ml and 1×10^3 paramecia/ml in a 1-ml depression slide at 25°C. The paramecia were observed under a Nomarski differential-interference-contrast (DIC) microscope 1 hr and 24 hr after mixing.

Cryopreservation of isolated H. recta

Isolated cells of the infectious form of *H. recta* were suspended in 10 mM Na, K-PB, containing 10% (v/v) glycerol in 1.5-ml serum tubes and immediately frozen at -85°C. Three, 10 and 127 days after freezing, the serum tubes were rapidly warmed with shaking in a water bath at 37°C. As soon as the ice was completely melted, the bacteria were washed once with 10 mM Na, K-PB, by centrifuging at 200 × g for 5 min at 4°C and suspended in the same buffer at 4°C until use.

Light microscopy

H. recta in the host micronucleus and those pushed out from the host cells were observed under a Nomarski DIC, phase-contrast and fluorescence optics (Olympus BH2-RFL) at a magnification of × 400 or × 1,000. To observe the infectious form of *H. recta*, cells isolated from the host homogenates by Percoll density centrifugation were air-dried on a glass slide, stained with 1 μg/ml of the DNA-specific fluorochrome, 4',6-diamidino-2-phenylindole (DAPI) dissolved in deionized water, and covered with a cover glass.

RESULTS

Morphology of the isolated infectious form of H. recta

The diameter of a micronucleus of *P. caudatum* is about 5-6 μm. However, when *H. recta* grew in the micronucleus, the nucleus became swollen (Fig. 1A). In some cases, the nuclear diameter reached more than 30 μm. When a cell was crushed between a slide and a cover glass, many holosporas in the micronucleus were pushed out from the cell (Fig. 1B). As shown in Fig. 1B, *H. recta* showed different morphologies in the life cycle as seen previously in *H. obtusa* and *H. elegans* (Fujishima *et al.*, 1990a; Görtz and Dieckmann, 1980); a reproductive short form (about 2.6 μm in length), an infectious long form (about 10 μm in length) and cells of intermediate lengths in the process of differentiation from the reproductive form to the infectious form. A division constriction was found only in the short form, indicating that reproduction by binary fission occurred only in this form, in agreement with the cases of *H. obtusa* and *H. elegans* (Fujishima *et al.*, 1990a; Görtz and Dieckmann, 1980). The fully elongated long form of this bacterium bent a little, and had a rounded end and a tapered end as described previously (Fokin, 1991).

The host homogenates were then centrifuged in 70% Percoll. Cells of the infectious form formed a band near the bottom of the centrifuge tube, between the density marker beads of 1.12 g/ml and 1.13 g/ml. On the other hand, the reproductive and intermediate forms formed another band near the top of the Percoll gradient with the host debris. This showed that the bacterium increases its density during differentiation from the reproductive form to the infectious form. Cells of the infectious form were collected, washed with 10 mM Na, K-PB, and stored at 4°C until use. As shown in Fig. 2A, the isolated infectious form of *H. recta* consisted of three distinct parts under a phase-contrast microscope: a dark part with a tapered end, a refractive part and a small dark tip at the rounded end as previously seen in *H. obtusa* and *H. elegans* (Fujishima and Hoshide, 1988; Görtz and Dieckmann, 1980). In *H. obtusa*

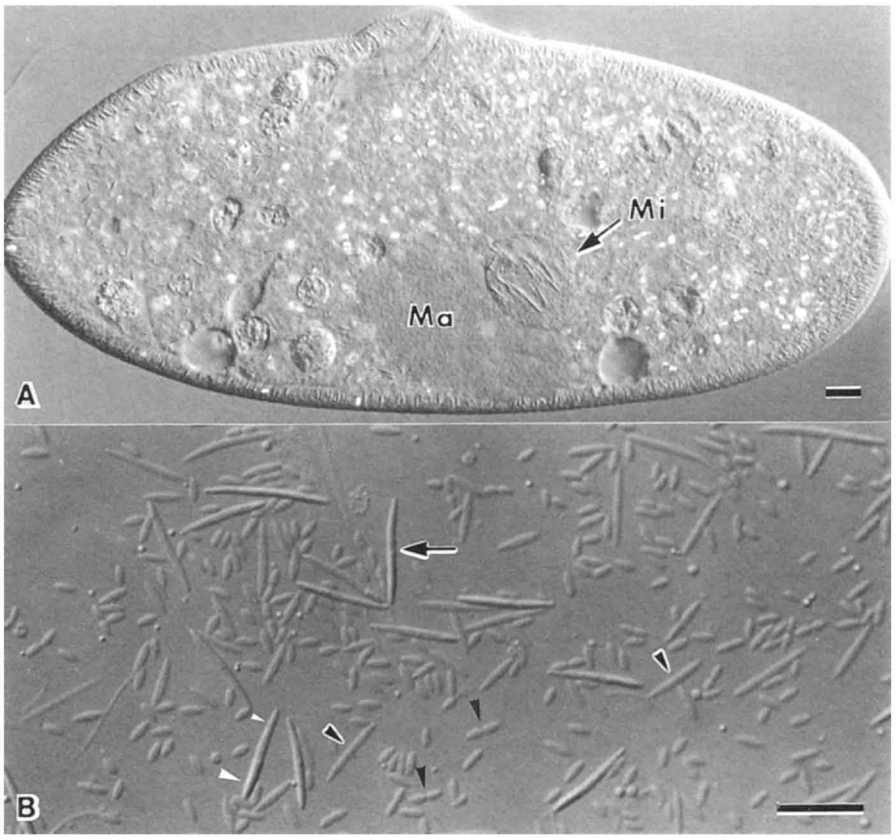

Fig. 1. Nomarski DIC photomicrographs of an *H. recta*-bearing *P. caudatum* cell (A) and *H. recta* released from a crushed host cell (B). The micronucleus-specific endosymbionts in A increased the size of the micronucleus (Mi). Note that many dividing reproductive short forms (black arrowhead), infectious long forms (arrow) and intermediate forms (white-rimmed arrowhead) between the reproductive and the infectious forms can be seen in B. Note that one end of the infectious form is rounded (large white arrowhead) but the other is tapered (small white arrowhead). Ma, macronucleus. Scale bar, 10 μm.

and *H. elegans*, it was found by transmission electron microscopy that the dark and refractive parts were the cytoplasmic and periplasmic regions, respectively (Wiemann, 1989). On the other hand, the small dark tip was composed of homogeneous materials with low electron densities, and it is known that this special tip is used to infect the host nucleus (Fujishima and Fujita, 1985; Görtz and Dieckmann, 1980). Therefore, the special tip seems to distinguish the two kinds of nuclei in the host and to invade the target nucleus. Furthermore, it was found that this heavy bacterial band did not contain fully elongated intermediate forms that had not yet developed the three distinct intracellular structures. Such the forms were observed in a less dense band together with the reproductive form and the host debris (data not shown). These results suggested that *H. recta* elongates to the length of the infectious form while it has the lower density of the reproductive form, and then increases its density as it begins to develop the three intracellular structures.

When isolated cells of the infectious form were stained with DAPI, the fluorescence appeared as a large cluster in the bacterium (Fig. 2C). This indicated that DNA is localized at a specific region of the infectious form. As shown in Fig. 2B-D, unlike the infectious forms of *H. elegans* (Görtz and Dieckmann, 1980) and *H. obtusa* (Fujishima and Hoshide, 1988; Fujishima *et al.*, 1990a, 1991) the DAPI-positive region of *H. recta* was apparently seen in the cytoplasmic region (dark region under a phase-contrast microscope) adjacent to the periplasm (refractive region under a phase-contrast microscope).

Infectivity and reproducibility of the isolated infectious form of H. recta

When isolated cells of the infectious form were added to paramecia at 25°C, they soon were engulfed by the host digestive vacuoles, and some of them escaped into the cytoplasm and invaded the host micronucleus. The first bacterium appeared in the micronucleus within 30 min. We could not observe infection of the macronucleus by this bacterium, indicating that the bacterium can distinguish the two kinds of host nuclei and infects only the micronucleus. It was found that the infected bacteria formed constrictions to differentiate into the reproductive short form in the *Paramecium* micronucleus within 18 hr after the infection.

Infectivity of H. recta *to other* Paramecium *species*

We examined whether the isolated infectious form of *H.*

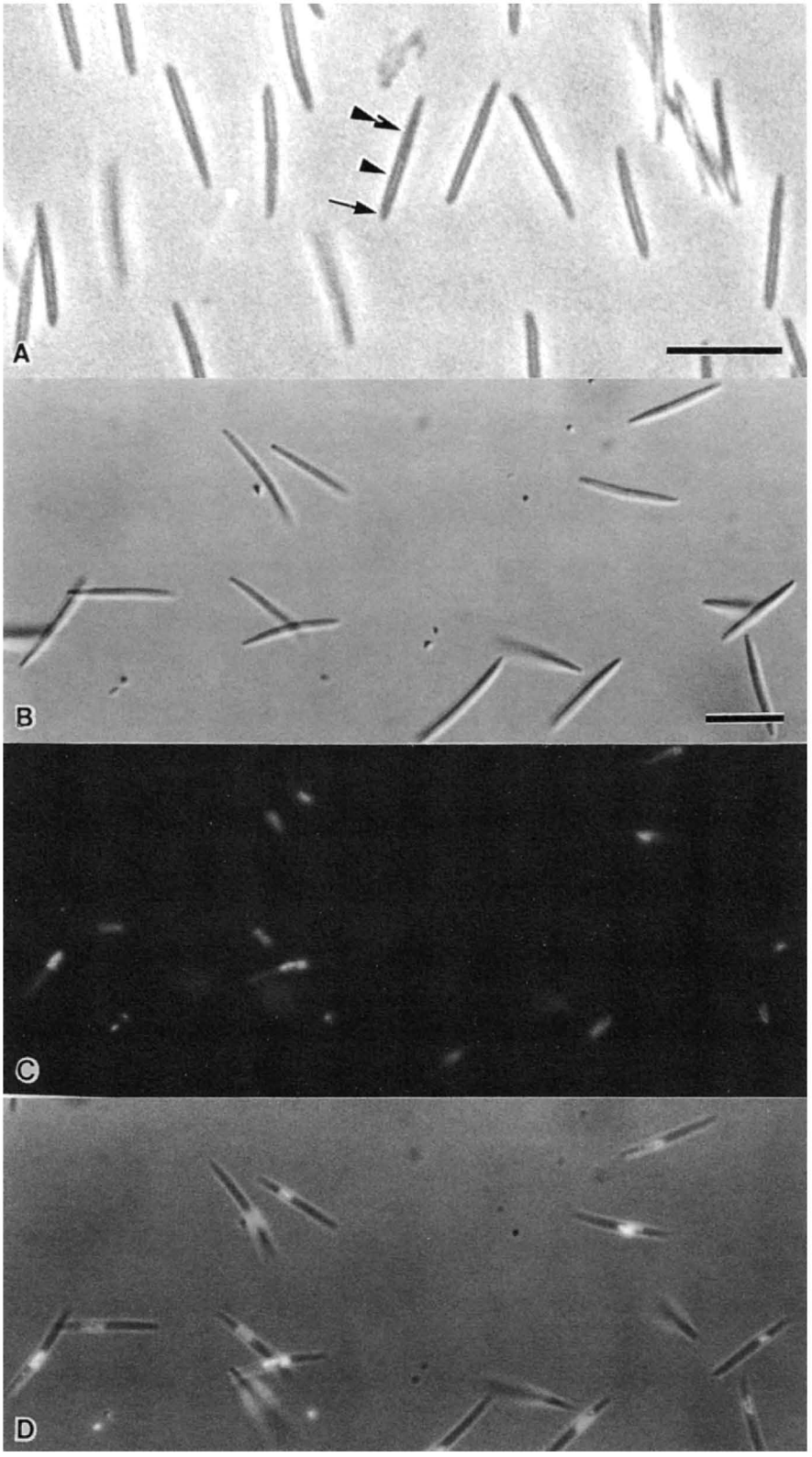

Fig. 2. Photomicrographs of isolated infectious forms of *H. recta*. Phase-contrast (A), Nomarski DIC (B), DAPI fluorescence (C) of B, and DAPI fluorescence and ordinary optics (D) of B. Note that the isolated infectious form consists of three parts: a dark part (double arrowhead), a refractive part (arrowhead) and a small dark-looking tip (arrow) in A. DAPI fluorescence is localized at the dark part near the border between the dark part and the refractive part. Scale bar, 10 μm.

recta also can infect other *Paramecium* species other than *P. caudatum*. The bacteria were mixed with cells of *P. multimicronucleatum* strains 49B and 53B, *P. bursaria* strain ET50-16 and *P. novaurelia* strain 91YB1-3, and the *Paramecium* cells were observed 1 hr and 24 hr after mixing under a Nomarski DIC microscope. *H. recta* were unable to infect these *Paramecium* nuclei in two trials for each species.

Cryopreservation of the isolated infectious form of H. recta

If cryopreservation of the isolated infectious form of *H. recta* is practicable, maintenance of this bacteria would be easy. Previously, we succeeded in the cryopreservation of the infectious form of *H. obtusa* at -85°C (Fujishima *et al.*, 1991). Using the same method, isolated *H. recta* cells of the infectious form were frozen in 10 mM Na, K-PB, containing 10% glycerol at -85°C. Three, 10 and 127 days after freezing, the bacteria were thawed, washed and mixed with paramecia to examine their infectivity and reproducibility after infection. In three cases, the bacteria were soon engulfed by the host digestive vacuoles after mixing. Some of them then escaped from the vacuoles to appear in the host cytoplasm, and infected the micronucleus within 1 hr after mixing just as did the non-frozen control bacteria. Within 24 hr after mixing, infection was observed in the majority of the *Paramecium* cells as it did in the control. We also confirmed that the bacteria that infected the host micronucleus differentiated into the reproductive form within 18 hr after the infection.

DISCUSSION

In a 70% Percoll density gradient, the infectious form of *H. recta* formed a band between the densities of 1.12-1.13 g/ml. In case of the infectious form of *H. obtusa*, its density was 1.16 g/ml (Fujishima *et al.*, 1990a). Thus, it was found that the density of the infectious form of *H. recta* was lighter than that of *H. obtusa*. In the present study, we did not isolate the reproductive form of this bacterium. However, as we succeeded in doing this with *H. obtusa* (Fujishima *et al.*, 1990a) it seems possible to isolate the reproductive form if micronuclei bearing the reproductive form are isolated first and then the nuclear homogenates are centrifuged in 40% Percoll.

The DAPI-positive region in the infectious form differs between the *Holospora* species examined. In *H. elegans*, the DAPI-positive region exactly corresponds to a dark region (cytoplasmic region) under a phase-contrast microscope (Görtz and Dieckmann, 1980). However, in *H. obtusa*, the DAPI-positive region coincides with a refractive region (periplasmic region) (Fujishima, 1986; Fujishima and Hoshide, 1988; Fujishima *et al.*, 1991). Because the refractive region corresponds to the periplasmic region, and because a narrow cytoplasmic extrusion had been observed in the periplasmic region of *H. obtusa* under an electron microscope (Görtz *et al.*, 1992), it seems that localization of DNA in this narrow cytoplasmic extrusion makes the refractive region DAPI-positive. In this study, we found that *H. recta* possesses a DAPI-staining pattern intermediate between those of *H. elegans* and *H. obtusa*. Namely, only a limited cytoplasmic region closely adjacent to the large periplasm showed DAPI-fluorescence. Furthermore, although *H. obtusa* had shown two spots with strong DAPI-fluorescence in the DAPI-positive region (Fujishima and Hoshide, 1988; Fujishima *et al.*, 1990a, 1991) as did *H. elegans* (Görtz and Dieckmann, 1980), *H. recta* did not show such spots. These observations indicate that *H. recta* differs from *H. obtusa* and *H. elegans* not only in morphology, but also in the localization of DNA in the infectious forms.

We also found that the timing of the formation of a constriction in the infectious form of *H. recta* differs from that of *H. obtusa*. *H. recta* formed the constrictions to differentiate into the reproductive form within 18 hr at 25°C, while *H. obtusa* did it between 32-34 hr after infection (unpublished data). Although the timing of the constriction formation in the early infection process has not yet been examined in other *Holospora* species, our data show that *H. recta* begins to form it within about half the time needed in *H. obtusa*.

Paramecium species can be classified morphologically into two groups: an "aurelia group" and a "bursaria group". The macronucleus-specific *H. obtusa* infects the macronucleus of not only *P. caudatum*, but also 14 species of the *P. aurelia* species complex and *P. multimicronucleatum*, although maintenance of the infected bacteria is achieved only in specific strains of *P. caudatum* (Fujishima and Fujita, 1985; Fujishima, 1986). All these *Paramecium* species belong to the "aurelia group". The *Paramecium* species belonging to the bursaria group (*P. bursaria*, *P. trichium*, *P. calkinsi* and *P. woodruffi*) were not infected by *H. obtusa* (Fujishima and Fujita, 1985; Fujishima, 1986). Therefore, it seemed that a property of the macronucleus, necessary for it to be recognized and infected by *H. obtusa*, was present not only in *P. caudatum* but also in closely related *Paramecium* species belonging to the "aurelia group". *H. recta*, like *H. obtusa*, could not infect *P. bursaria*, but unlike *H. obtusa*, it also could not infect *P. multimicronucleatum* or *P. novaurelia*. This indicates that the species-specificity of the infectivity of *H. recta* is more strictly controlled than that of *H. obtusa*. This suggests that although macronuclear envelope specific substances distinguished by *H. obtusa* may be present in various species belonging to the "aurelia group", but micronuclear envelope specific substances distinguished by *H. recta* may be present only in *P. caudatum* or in very limited species if any. On the other hand, infectious forms of *Holospora* species have a structurally differentiated special tip at the one end (Fujishima and Hoshide, 1988; Fujishima *et al.*, 1990b) and the bacteria always penetrate the host target nuclear envelope with this special tip first. This suggests that substances at this tip have an ability to distinguish some differences between two kinds of the host nuclei and to penetrate the nuclear envelope. Although the substances supposed to be present at the special tip are not yet detected, it may be possible if the isolated infectious forms are used as antigens for obtaining of the monoclonal antibodies against the special tip.

In our previous study (Fujishima *et al.*, 1991), we

succeeded in the cryopreservation of the isolated infectious long form of *H. obtusa* at -85°C without losing their infectivity or reproducibility. Using the same method in the present study, we also succeeded in the cryopreservation of *H. recta*. Although it is not clear how many days the bacterium frozen at -85°C can maintain its infectivity and reproducibility, this duration may be prolonged with the use of liquid nitrogen. Cryopreservation made it possible preserve this bacterium for a long time without the need for laborious culture procedures.

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