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# Endonuclear symbiotic bacteria *Holospora* species distinguish the host nuclear envelopes

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**Abstracts**

The Gram-negative bacterium *Holospora* species can distinguish two kinds of nuclei, a macro- and a micronucleus, of the ciliate *Paramecium* species. To know mechanism how *Holospora* species can distinguish their target nuclear envelopes, monoclonal antibodies specific for the outer membranes of the macronucleus-specific *H. obtusa* and the micronucleus-specific *H. undulata* were developed. Indirect immunofluorescence microscopy showed that the outer membrane substances extracted from SDS-PAGE gels of each *Holospora* species bound to their target nuclear envelopes, when the extracts were mixed with isolated macro- and micronuclei. Immunoblots showed that relative molecular masses of the antigens were 16 kDa in *H. obtusa* and 13 kDa in *H. undulata*. These antigens were proteinase K resistant and unstained with Coomassie brilliant blue-R250 or with ordinary silver staining. However, these antigens were stained with silver for bacterial lipopolysaccharide. Our results showed that nucleus-specific infection of *Holospora* species was controlled by an affinity between lipopolysaccharides of the outer membranes of the infectious forms of *Holospora* species and the unknown receptor substances on the nuclear envelopes of the host cell.

## Introduction

The Gram-negative bacteria *Holospora* species are endonuclear symbionts of the ciliate *Paramecium* species (Fokin et al., 1996), and are belonging to $\alpha$ subclass of *Protea-*

*bacteria* from 16S rRNA-based phylogenetic tree (Amann et al., 1991). This bacterium grows by binary fission of the reproductive short form (1.5-2 μm in length) when the host cell is growing, but when the host cell starves, then the bacterium ceases growth and differentiates into the infectious long form (13 μm in length) (Fujishima et al., 1990a). During this differentiation, the bacterium forms a cytoplasmic region comprising half the cell length and a large periplasmic region with a small special tip. At this time, the bacterium acquires not only its infectivity but also its resistance against various chemical and physical factors (Fujishima et al., 1991). The bacterium also changes its morphology of the outer membrane during this differentiation (Fujishima et al., 1990b). When the infectious forms were isolated from the host homogenates and mixed with paramecia, they were soon engulfed into digestive vacuoles. Some of the bacteria then escaped from the vacuoles, appeared in the cytoplasm, and within 15 min infected their target nucleus by first penetrating the nuclear envelope with the special tip. Thereafter, they differentiated into the reproductive short forms about 48 h after the infection (Kawai and Fujishima, 2000). These observations suggest that either the outer membrane or special tip region of the infectious form may have an important role in recognition or in invading their target nucleus. The present study was intended to elucidate the mechanism how the infectious form of *Holospora* species distinguish their target nucleus.

## Materials and Methods

*H. obtusa*-bearing and non-bearing strains RB-1 (mating type E, syngen 4) and *H. undulata*-bearing strain PM-3 of *P. caudatum* were cultivated in lettuce juice medium at 25 °C (Hiwatashi, 1968). The infectious and reproductive-forms of *H. obtusa* were isolated by Percoll density gradient centrifugation as described previously (Fujishima et al., 1990a). The isolated bacteria were stored at - 85 °C until use. Monoclonal antibodies were obtained by routine procedures according to Galfre and Milstein (1981) with isolated infectious-forms of *H. obtusa* and *H. undulata* as antigens. Nuclei were isolated by

sucrose density gradient centrifugation (Freiburg, 1985; Fujishima et al., 1991). Isolated *Holospora* or *Paramecium* nuclei were fixed with 4% (w/v) paraformaldehyde for 1 h, washed two times with phosphate-buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.1 mM $NaHPO_4 \cdot 12H_2O$, 1.47 mM $KH_2PO_4$, pH 7.2) for 15 min each and incubated in primary monoclonal antibody-containing culture supernatant for 1 h at room temperature. The samples were washed 3 times with PBS for 15 min each and incubated in fluorescein-isothiocyanate (FITC)-conjugated secondary antibody for 1 h at room temperature. Observations were carried out with fluorescence optics (Olympus, BH2-RFL) at a magnification of 400X. Cell pellets of *Holospora* were lysed by boiling with Laemmli's lysis buffer. The proteins in the lysate were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each lane was loaded with lysates of about $5 \times 10^7$ bacteria. The gels were then stained with a silver or Coomassie brilliant blue R 250. After SDS-PAGE, proteins on the gel were transferred to an Immobilon-P membrane. The membrane was then incubated overnight at 25°C with primary monoclonal antibody -containing culture supernatant containing 5% (w/v) skim milk, then stained using Vectastain ABC-AP kit. For extraction of substances from specific bands of the gel, regions of the non-stained gel corresponding to the bands were cut off, put in a dialyzing membrane tube with 1-2 ml of an electrode buffer for native-PAGE (30% [v/v] glycerine, 6% [w/v] Tris, pH 6.8). The dialyzing tube was suspended in an electrode buffer for native-PAGE in a protein recovery apparatus (Nihon Eido, NA1710), and substances were electro-eluted from the gel fragments at 100 V constant voltage for 1 h. The tube was then dialyzed against deionised water for overnight at 4°C.

**Results and Discussion**

We developed monoclonal antibody IR-4-1specific for *H. obtusa* and Rlu-1 specific for *H. undulata* (Kawai and Fujishima, 1997). Both antibodies labelled outer membranes of the reproductive and the infectious forms of each bacterium by indirect immunofluo-

rescence microscopy. Fig. 1 shows FITC-fluorescence of outer membranes of the reproductive and the infectious form of *H. obtusa*. FITC fluorescence did not appear on outer membranes of reproductive and infectious forms of *Holospora* if the bacteria were not treated with the primary antibody. Also, indirect immunofluorescence microscopy showed that IR-4-1did not label *H. undulata*, and Rlu-1 did not label *H. obtusa* (data not shown). These indicate that FITC-fluorescence in Fig. 1 is not due to non-specific reaction of the antibodies used. Outer membrane of *Holospora* species is very hard, and does not pass antibodies to enter into the periplasm and the cytoplasm even after fixation and extraction for ordinary indirect immunofluorescence microscopy (Dohra and Fujishima, 1999). Therefore, Fig. 1 indicates the epitopes are exposed outside the outer membranes.

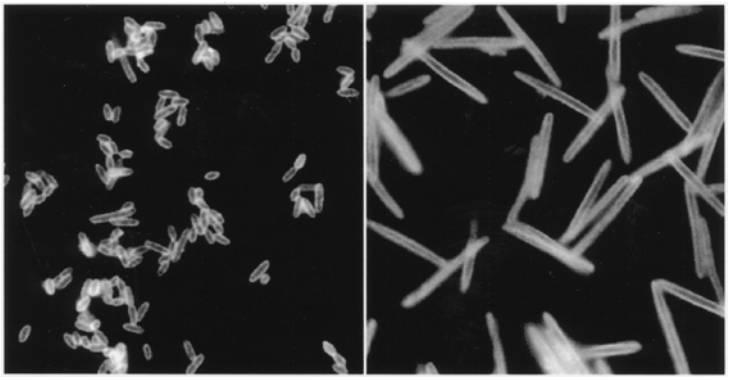

**Figure 1**: Indirect immunofluorescenece micrographs of *H. obtusa* with a monoclonal antibody IR-4-1 specific for outer membrane. Left, reproductive forms. Right, infectious forms.

Using these two monoclonal antibodies, outer membrane substances were extracted from SDS-PAGE gels. When the outer membrane substances were mixed with isolated macro- and micronuclei of *P. caudatum* and observed by indirect immunofluorescence microscopy with the two kinds of antibodies, it was confirmed that the outer membrane substances bound only to the target nuclear envelopes (Kawai and Fujishima, 1999). Relative molecular masses of the antigens that bound with the target nuclear envelopes were 16kDa in *H. obtusa* and 13kDa in *H. undulata*. These antigens were resistant against proteinase K and could not be stained with Coomassie brilliant blue-R250 and ordinary silver staining. However, the antigens were stained by silver for bacterial

lipopolysaccharide, indicating that the substances of both *Holospora* species were lipopolysaccharides of their outer membranes (Kawai and Fujishima, 1999). Thus, our data show that nucleus-specific infection by *Holospora* species is controlled by an affinity between a sensor substance of the outer membrane of the infectious form and an unknown receptor substance of the target nuclear envelope (Fig. 2).

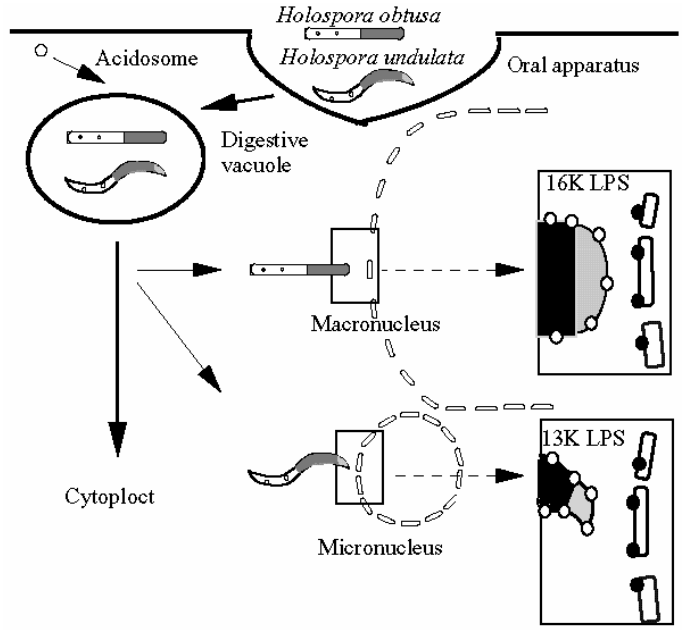


**Figure 2**: Schematic representation of infection process in *H. obtusa* and *H. undulata*. When infectious forms are mixed with paramecia, the bacteria are ingested into the host digestive vacuole. Some of the infectious forms escape from the acidified vacuoles to appear in the cytoplasm. The bacteria distinguish their target nuclear envelopes with an affinity between their sensor substances on outer membranes (open circles) and unknown receptor substances on the target nuclear envelope (closed circle). The sensor substance of *H. obtusa* is a lipopolysaccharide of 16 kDa, and that of *H. undulata* is a lipopolysaccharide of 13 kDa. After that, the bacteria penetrate the nuclear envelopes by their special tip first.

Two kinds of monoclonal antibodies, IR-4- and Rlu-1, labelled outer membranes of the reproductive forms of both *Holospora* species by indirect immunofluorescence microscopy. Molecular masses of the antigens were also similar with those of the infectious forms by immunoblots. However, the antigens extracted from SDS-PAGE gels could not bind to their target nuclear membranes of the isolated nuclei (unpublished data). This suggests that lipopolysaccharides of the reproductive forms acquire an ability to bind their target nuclear envelopes by partial modifications during differentiation to the infectious form.

Infectious forms have a structurally differentiated special tip at the one end (Fujishima and Hoshide, 1988; Fujishima et al., 1990b; Kawai and Fujishima, 2000) and the bacteria always penetrate the host target nuclear envelope with this special tip first. Therefore, it had been expected that substances at this tip should have an ability to distinguish

some differences between two kinds of the host nuclei and to penetrate the nuclear envelope. However, present study showed that *Holospora* distinguished the host nuclei by lipopolysaccharides, and the lipopolysaccharides were present not only at the special tip surface but also at the other surfaces too. This indicates that the special tip may have a function to penetrate the target nuclear envelope.

Two kinds of the host nuclei, a macro- and a micronucleus are originated from a common fertilization nucleus. If the receptor substances against the sensor substances of *Holospora* species are present outside each nuclear envelopes, we can expect to develop monoclonal antibodies against each nuclear envelope. Recently, we succeeded to develop seven kinds of monoclonal antibodies specific for the macronuclear envelope of *P. caudatum* (unpublished data). The receptor substances may be involved in these macronuclear envelope-specific antigens.

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