Two Different Size-Distributions of Engulfment-Related Vesicles Among Symbiotic Protists of the Lower Termite, *Reticulitermes speratus*

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We analyzed symbiotic protistan engulfment systems using the method of fluorescent imaging. Symbiotic protists can be divided into two protistan groups, Type S (protists that contain uniformly small and spherical vesicles) and Type L (protists that contain vesicles that vary in both size and form). This classification corresponds not to the taxonomic classification, but to the micro-habitats of the protists. Our results suggested that these protists might have adapted to different resources in the hindgut environment of lower termites.

Key words: termite, symbiotic protists, endosome, phagosome

In terms of phylogeny, termites are divided into higher and lower termites, with all of the lower termites hosting symbiotic protists (Phylum Parabasalia and Order Oxymonadida) in their hindgut^{3,4,10}. Previous defaunation and tracer studies have indicated that the lower termites are incapable of utilizing cellulose without these protists^{2,5}, suggesting that the symbiotic protists degrade the cellulose in ingested wood particles. Further, microscopic observations have indicated that these protists selectively engulf only ingested particles that contain cellulose^{9,11,12}. It thus appears that the symbiotic protists have specific machinery to engulf the wood materials ingested by termites.

This capability to engulf wood materials selectively, suggests that the symbiotic protists in the hindgut of lower termites play a special role in the cellulose decomposition process. However, several scientific questions remain unanswered. While Yamaoka and his colleagues demonstrated that *Trichonympha agilis* was capable of engulfing and degrading large cellulose particles⁹, no data is available on how other small protists engulf particles or the form and material composition of the particles they engulf. Further,

some researchers have observed an intriguing segregation of the habitat of symbiotic protists in the hindguts of termites^{11,12}. By analyzing the engulfment process in each protist in the hindgut protistan community, we can speculate more accurately on the reasons for this segregation and the ecological functions of different protist species.

In this study we analyzed the engulfment system of symbiotic protists in the hindgut of the lower termite *Reticulitermes speratus* using fluorescent imaging analysis. The results indicated that the symbiotic protists can be divided into two types based on the size of engulfment-related vesicles. One possible explanation is that two different engulfment systems developed with the protistan adaptation to resources available in the gut environment of the termite.

Materials and Methods

Collection and maintenance of the termites

Reticulitermes speratus was collected from the Tanzawa range, Kanagawa, Japan and fed on wet cellulose powder in 6 cm plastic dishes at room temperature for at least 3 days.

Fluorescence Microscopy

For the first two days, *R. speratus* were initially placed on

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an artificial diet containing FM4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl)hexatrienyl)pyridinium dibromide) and FITC-dextran (cellulose powder 0.05 g, 6 mg/ml FITC-dextran 50 μ l, 16 mM FM4-64 25 μ l, de-ionized water 75 μ l) in 6 cm plastic dishes at room temperature^{7,8)}. From the third day, the termites were fed an artificial diet containing FITC-dextran only (cellulose powder 0.05 g, 6 mg/ml FITC-dextran 50 μ l, water 100 μ l) in 6 cm plastic dishes at room temperature for 3–10 days. For observation, we selected 10 pseudergates and used forceps to remove their guts en masse from the posterior ends of their bodies. Once placed in a suspension of 100 μ l of ice-cold solution U (37 mM NaCl, 5.13 mM $C_6H_5Na_3O_7 \cdot H_2O$, 13.1 mM KH_2PO_4 , 9.1 mM $NaHCO_3$, 0.56 mM $CaCl_2 \cdot 2H_2O$ and 0.2 mM $MgSO_4 \cdot H_2O$) the gut sections were gently torn open to release their contents. After the suspension was cen-

trifuged at 500 rpm and 4°C for 3 min, the pelleted protists were washed 3 times with 100 μ l of ice-cold solution U and fixed with 2% paraformaldehyde (PFA), 2.5% glutaraldehyde, and solution U for 15 min on ice. The fixed protists were washed 3 times in ice-cold solution U and embedded in VECTASHIELD mounting medium (Vector laboratories Inc. Burlingame, U.S.A.). The samples were observed with a BIORAD model Radiance2100 confocal laser scanning microscope. For FM4-64, the samples were observed with a 488 nm excitation wavelength laser and fluorescence emission filter 600LP. For FITC-dextran, the samples were observed with a 488 nm wavelength excitation laser and fluorescence emission filter HQ515/30. The digital images obtained were recorded in the "Bio-Rad Confocal Pic File" (.pic) format and analyzed by computers.

Fig. 1. Fluorescence microscopic analysis of uptake vesicles. (a) *Dinenympha exilis* (b) *Dinenympha porteri* (c) *Pyrsonympha grandis* (d) *Teranympha mirabilis* (e) *Trichonympha agilis*. Extracellular material (FITC-Dextran stain, green) and engulfment-related vesicles (FM4-64 stain, red) are observed. The arrowhead denotes a small globular form vesicle. The arrow denotes vesicle of a various size and form. Bars, 10 μ m.

Results and Discussion

FITC-dextran and FM4-64 fluorescence probes to distinguish and visualize the uptake pathway

We performed fluorescent imaging of engulfment-related vesicles using FITC-conjugated dextran (FITC-dextran) and FM4-64. Both fluorescent dyes were supplied with the termite diets. FITC-dextran, a high molecular weight polymer that emits green fluorescence, is engulfed with food as an environmental fluid and remains within the food vacuoles with engulfed materials. Once within the vesicles, its high molecular weight prevents it from diffusing back out. FM4-64, a red fluorescent material, selectively stains the outer plasma membrane and engulfment-related vesicles due to its inability to pass through the outer membrane. When we observed a green signal with a red surrounding signal in our experiment, it indicated engulfed materials and engulfment-related vesicles.

We used this fluorescent staining method to observe all of the protists inhabiting the hindgut of *R. speratus*⁶⁾. According to our results, the engulfment-related vesicles in the Order-Oxymonadida protists had mainly small globular vesicles measuring 1–2 μm in diameter (Fig. 1, Panel a, b, c). When these vesicles were observed by differential inter-

ference microscopy, wood particles were not detected. This result suggested that the Oxymonads mainly engulfed fluidal material (data not shown). In the Oxymonads, *Pyrrsonympha grandis*, however, differential interference microscopy revealed different sized globular vesicles with wood particles (data not shown). In the case of two Parabasalid protists, *Trichonympha agilis* and *Teranympha mirabilis*, the vesicles were variable both in size and in form (Fig. 1, Panel d, e). Differential microscopic observation indicated that these protists also engulfed wood particles (data not shown)

To confirm the microscopic observations, we measured the sizes of the vesicles (Fig. 2). A size plotting chart indicated that the Oxymonad *Dinenympha exilis* and *Dinenympha porteri* used small globular vesicles to engulf extracellular materials. In the case of *D. exilis*, no vesicles over 5 μm in size were observed, while in *D. porteri*, no vesicles over 10 μm in size were observed and almost all vesicles were smaller than 5 μm . In the case of the Parabasalids and the Oxymonad *Pyrrsonympha grandis*, however, the extracellular materials were engulfed by vesicles of various sizes and forms. In contrast with *D. exilis* and *D. porteri*, these three protists contain huge vesicles over 10 μm in size and those vesicles include wood particles (data not shown). Crown eukaryotes possess endosomes and phagosomes to

Fig. 2. Quantitative analysis of uptake vesicle size. The lengths of the major axis and minor axis of engulfment-related vesicles were measured. Measured protistan species are *Dinenympha exilis* (55 vesicles), *Dinenympha porteri* (72 vesicles), *Pyrrsonympha grandis* (59 vesicles), *Teranympha mirabilis* (85 vesicles) and *Trichonympha agilis* (141 vesicles). Vesicles over 5 μm in size, that include wood particles, are indicated by red dots.

engulf extracellular materials. The endosomes are all the same size and surrounded by coated protein. The phagosomes are variable in size and can engulf large extracellular materials. The results of our observation perhaps suggest that the small globular vesicles of the Oxymonads *Dinenympha exilis* and *Dinenympha porteri* have mainly endosome-like vesicles, while the Parabasalids and Oxymonad *Pyrsonympha grandis* have variably sized and shaped vesicles such as phagosome-like vesicles. This hypothesis should be confirmed by immuno-fluorescent analysis using an anti-clathrin antibody that cross reacts with symbiotic protistan proteins.

What kind of symbiotic system does each symbiotic protist employ?

Thus, symbiotic protists of *R. speratus* can be divided into two types based on the mechanisms by which they engulf extracellular particles. The first type, *Dinenympha exilis*, *Dinenympha porteri*, and related Oxymonads of a relatively small size, use small uniform vesicles and take up only fluidal material. The second type, the Parabasalids and the large Oxymonads *Pyrsonympha grandis*, use vesicles of various sizes and forms and can take up wood particles. We defined these two protist types as Type S and Type L, respectively.

This classification corresponds with the micro-habitats of these protists in the hindgut of *R. speratus*. Some researchers have suggested that large protists reside on the esophagus-side while small protists reside on the anus-side in the hindgut¹¹⁾. This distribution suggests that the Type L protists play a principal role in digesting particle wood components on the esophagus-side in the hindgut while the Type S protists digest the unused, possibly fluidal, material on the anus-side. If this is true, these two groups of protists participate in a “resource segregation” system to utilize all the material components of ingested dead wood.

Interestingly, this classification based on engulfment vesicles does not correspond with the taxonomic classification, because the same taxonomy unit-Oxymonad was divided into two groups, Type S and Type L. Perhaps, the “resource

segregation” system evolved with adaptation to different resources in the hindgut environment, as mentioned above. Further analyses on the cargo of each type of vesicle will prove this “resource segregation” hypothesis.

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