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Structural Similarity of the Membrane Envelopes of Rhizobial Bacteroids and the Host Plasma Membrane as Revealed by Freeze-Fracturing

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The freeze-fracture technique was used to study the host plasma membrane and the membrane envelope of bacteroids in rhizobial root nodules of three host-rhizobium combinations. In all three combinations studied, the membrane envelopes of bacteroids are structurally similar to their host plasma membrane. However, the membrane appears to be reversed, because the number and arrangement of particles in the outer fractured face (face A, concave) and in the inner fractured face (face B, convex) of the host plasma membrane are seen, respectively, in the inner fractured face (face B, convex) and in the outer fractured face (face A, concave) of the membrane envelope of the bacteroids at an early stage. This reversion of the membrane surface is consistent with the hypothesis that the membrane envelopes of bacteroids are derived from the host plasma membrane during endocytotic engulfment.

A free-living rhizobium and a rhizobium in the intercellular space have a plasma membrane inside a cell wall, but the rhizobium in situ in the cytoplasm of a root nodule cell has an additional membrane outside its cell wall (5, 7, 10). This membrane is generally referred to as the membrane envelope. The origin of the membrane envelope has not been established (10, 16). There are several major hypotheses for the biogenesis of this membrane envelope. One hypothesis states that rhizobia acquire their membrane envelopes from the host plasma membrane in the course of their entry into the host cells (1, 4, 6). Others state that the membrane is derived from endoplasmic reticulum (8, 9, 12), outer nuclear membrane (13), or de novo (3, 7).

Recently, Tu (16) demonstrated the relationship of the host plasma membrane and the membrane envelopes of rhizobial bacteroids by histochemical localization of adenyl cyclase, a primary plasma membrane-bound enzyme (14, 16). Electron-dense reaction products resulting from adenyl cyclase activity were localized on the outer surface of the host plasma membrane and also on the inner surface of the membrane envelope (16). The localization of the electron-dense products on the inner surface was interpreted as an indication that rhizobium enters the host cell by endocytosis. As a result, the membrane surfaces of the membrane envelope are the reverse of the plasma membrane surfaces (16). If this interpretation is correct, the following conditions should be met: (i) the initial membrane envelope and the host plasma membrane should be structurally similar; (ii) the outer surface of the host plasma membrane should be equivalent to the inner surface of the membrane envelope; and (iii) the similarity between the membrane envelope of the bacteroids and the host plasma membrane should be applicable to various *Rhizobium* species which enter by endocytosis.

Freeze-fracturing is a unique way to study the structure of various membranes, because it exposes fractured faces which are not discernible by negative staining and thin sectioning (2, 11, 15). When a unit membrane is fractured, the inner half and the outer half can be easily identified by the nature of the fracture (such as convex or concave) and the density and distribution of particles on each fractured face. This technique is used in this investigation to elucidate the origin of the membrane envelopes in three species of root nodules arising from the symbiosis between *Rhizobium meliloti* (alfalfa), *Rhizobium trifolii* (clover), and *Rhizobium japonicum* (soybean).

MATERIALS AND METHODS

Inocula. *R. meliloti* Dangeard, *R. trifolii* H.K.C., and *R. japonicum* (Kirchner) Buchanan were maintained on agar slant in test tubes. The agar medium consisted of 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4$, 0.1 g of

NaCl, 3 g of yeast extract, 10 g of mannitol, and 12 g of agar per liter of water. For inocula, rhizobia were grown in liquid medium (i.e., the agar medium without agar) in flasks and incubated in a 25 C water bath shaker for 2 days. The rhizobial cultures were diluted to a concentration of 10^4 rhizobium cells per ml with sterilized water before inoculation.

Plants. Seeds of alfalfa (*Medicago sativa* L., "alfa"), clover (*Trifolium hybridum* L.) and soybean (*Glycine max* (L.) Merr. 'Amsoy') were germinated separately in petri plates for 3 days, and transplanted to 14-cm plastic pots containing soil-sand-peat at 2:1:1, one plant per pot. Seedlings of alfalfa, clover, and soybean were inoculated with *R. meliloti*, *R. trifolii*, and *R. japonicum*, respectively. Inoculations were made by applying 250 ml of rhizobial suspension containing 10^4 rhizobium cells per ml each pot. After inoculation, the plants were maintained in a 22 ± 1 C growth chamber programmed for 10 h of darkness and 14 h of light with a light intensity of 26,000 lx measured at the bench level.

Freeze-fracturing. Root nodules were sampled 3 weeks after inoculation. The root nodules were fixed for 3 h in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, followed with two washes, 15 min each, and then placed in 25% glycerol overnight at 4 C. The materials were frozen in Freon-22, quickly transferred to and stored in liquid nitrogen, and processed in Balzers BA 360 M high vacuum freeze-etch unit as previously described (15). The materials were freeze-fractured at −100 C and shadowed with platinum and carbon at about 10^{-6} torr vacuum pressure. The replicas were cleaned, picked up on formvar-coated grids, and examined in an electron microscope at 80 kV.

RESULTS AND DISCUSSION

General remarks. Only the freshly infected cells were examined to observe the structure of the membrane envelope soon after engulfment before there was much opportunity for it to lose its native structure.

It is known that the faces of a fractured membrane are not true surfaces of the membrane, because a tripartite unit membrane usually fractures along the hydrophobic layer which is sandwiched in between the two hydrophilic layers (2). For this reason, the fracture of a unit membrane such as a plasma membrane usually gives rise to two fractured faces. One is on the outer half (facing the cell wall) and the other is on the inner half (facing the cytoplasm) of the plasma membrane. The former and the latter are conventionally referred to as face A and face B. Face A is usually seen as a concave structure and face B is revealed as a convex structure, since a cell is normally somewhat spherical in shape.

Since the basic features of all three *Rhizobium*-host combinations are essentially the same, micrographs of only the *Rhizobium meliloti*-alfalfa combination are presented.

The plasma membranes of the bacteroidal cells. The plasma membranes of the bacteroidal cells of alfalfa, clover, and soybean have different densities of particles in their fractured faces (Table 1). The total particle density of both fractured faces of the plasma membrane is characteristic with a species of root nodule. It is highest in the soybean root nodule, intermediate in the alfalfa root nodule, and lowest in the clover root nodule (Table 1).

Regardless of the host, face B has a higher particle density than face A. This observation is consistent with other reports on the plasma membranes of various organisms (2). It should be noted that the arrangement of particles in face A of all 3 species of bacteroidal cells appears to be random (Fig. 1). However, in face B, particles are arranged in a network (Fig. 2).

The membrane envelopes of the bacteroids. The membrane envelopes of the bacteroids are also unit membranes. Therefore, they also have two fractured faces, i.e., a concave-fractured face (face A) and a convex-fractured face (face B) (Fig. 3). However, contrary to the plasma membranes of the bacteroidal cells, face A has many more particles than face B in all three species of bacteroids (Table 1). The particle density of both faces of the membrane envelope in each species of bacteroid is close to that of the plasma membrane of the host cell (Table 1).

In the membrane envelopes of all three species of bacteroids, face A has a higher particle density than face B. It should be noted that the number of particles on faces A and B of the membrane envelopes of all three species of bacteroids approximates the number of particles on faces B and A of the plasma membrane, respectively (Table 1). Moreover, on the membrane envelopes of the bacteroids, particles are distributed randomly in face B, whereas those in face A are arranged in a network (Fig. 4),

TABLE 1. *Particle distribution on the fractured faces of the host plasma membrane and the membrane envelope of bacteroids in soybean (R. japonicum), alfalfa (R. meliloti), and clover (R. trifolii) combinations*

Host	Soybean		Alfalfa		Clover	
Fractured face	Face A[a]	Face B[a]	Face A[a]	Face B[a]	Face A[a]	Face B[a]
Host plasma membrane	319[b]	1585	502	1080	415	648
Membrane envelope	1646	303	1150	522	620	389

[a] P values ($P < 0.001$) indicate the level of statistical significance obtained from t-tests.

[b] Average particle number in 1 μm^2 area derived from four replicated particle counts.

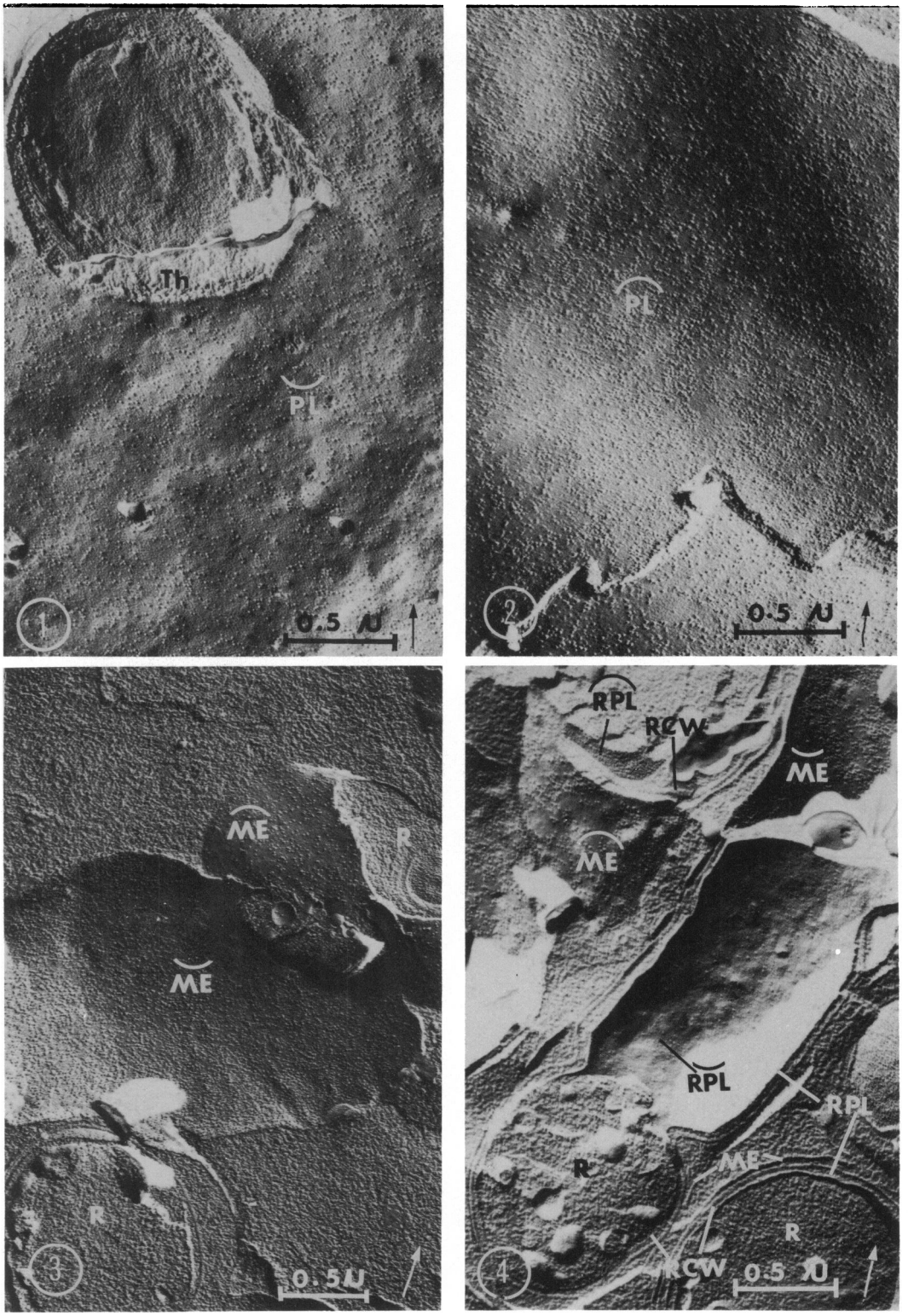

FIG. 1–4. *Thin sections of alfalfa root nodule tissue cell showing the concave- and convex-fractured faces of the host plasma membrane and of the membrane envelopes of bacteroidal cells. The abbreviations and signs used in these figures are: ME, membrane envelope; PL, host plasma membrane; R, rhizobium; RCW, rhizobial cell wall; RPL, rhizobial plasma membrane; Th, infection thread;* ⌒, *convex-fractured face; and* ⌣, *concave-fractured face. (1) Concave-fractured face of the host plasma membrane. The rounded hole is a cut neck of an infection thread. (2) Convex-fractured face of the host plasma membrane. (3) Concave- and convex-fractured faces of the membrane envelopes of a bacteroidal cell at the early stage of rhizobial infection. (4) Comparison of the concave- and convex-fractured faces of the membrane envelope and those of the plasma membrane of the bacteroids.*

which is the reverse of the distribution seen in the plasma membrane of the host.

According to the particle density and the pattern of particle distribution, faces A and B of the membrane envelope resemble faces B and A of the host plasma membrane, respectively. This observation is consistent with the hypothesis that the rhizobium enters the host cell endocytotically and derives its membrane envelope from the host plasma membrane (1). This is also consistent with previous observations using adenyl cyclase as an enzyme marker to demonstrate the relation of the host plasma membrane to the membrane envelope of the bacteroids (16).

The present study, using three different host-rhizobium combinations, provides further evidence in support of the hypothesis that the membrane envelope of bacteroids originates from the host plasma membrane.

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