JOURNAL OF BACTERIOLOGY, Sept. 1974, p. 986-991

Vol. 119, No. 3
Printed in U.S.A.

Relationship Between the Membrane Envelope of Rhizobial Bacteroids and the Plasma Membrane of the Host Cell as Demonstrated by Histochemical Localization of Adenyl Cyclase

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Received for publication 24 April 1974

By using adenyl cyclase as a marker enzyme, the relationship between the membrane envelope of the bacteroids of rhizobia and the plasma membrane of the host cell was demonstrated histochemically. Electron-dense deposits were found on the outer surface of the plasma membrane of the host cell and on the inner surface of the membrane envelopes of the bacteroids, but not in vacuole membranes, endoplasmic reticula, Golgi apparatus, and mitochondrial membranes. The results suggest that the membrane envelopes of the bacteroids are closely related to the host plasma membrane, and that entry of the bacteroids into the cytoplasm is in a manner similar to endocytosis.

Root nodules of alsike clover (*Trifolium hybridum* L.) develop as a result of rhizobial infection. The rhizobia (*Rhizobium trifolii*) enter the host root system through root hairs and travel through the middle lamella and intercellular spaces towards the cortical cells. Once they reach the cortical cells, the rhizobia force the host cell wall inward to form a finger-like projection, termed an infection thread. Later, they enter the cytoplasm through a crack at the tip of the infection thread. Rhizobia, in vitro and in the infection thread, have their own plasma membrane inside their cell wall (6). However, after entering the host cytoplasm, an additional membrane, referred to as a membrane envelope, is observed outside the cell wall. The origin of the membrane envelopes is unclear, due to the fact that all hypotheses have so far been derived from observations on regularly prepared thin sections. Some researchers speculated that the membrane envelopes of the bacteroids originated from the plasma membrane of the host cell (1, 5, 6). Others, however, suggested that the membrane envelopes originated from endoplasmic reticulum (7-9), the outer nuclear membrane (10), or de novo (4, 7, 8).

If the membrane envelopes of the bacteroids originate from the host plasma membrane (1, 5, 6), then, after the bacteria are released from the infection thread, they may enter the host cell in a manner similar to endocytosis. If this is the case, then the membrane envelopes and the host plasma membrane should have some characteristics in common.

Adenyl cyclase is a membrane-bound enzyme and is primarily associated with the plasma membrane (11, 13-15). Since this enzyme can be localized histochemically (12, 15), it has been used as a marker enzyme to study the relationship between the membrane envelopes of the bacteroids and the plasma membrane of the host cell.

MATERIALS AND METHODS

Seeds of alsike clover (*T. hybridum* L.) were germinated on moistened filter paper in a petri dish. Seedlings at their first trifoliolate stage were transplanted into 6-inch (ca. 15.2-cm) pots (two plants per pot) filled with an autoclaved soil mixture consisting of sand, peat, and loam in 1:1:2 proportions. These potted plants were watered with 250 ml of rhizobial suspension per pot containing 10^8 rhizobia/ml (*R. trifolii*). Inoculated plants were kept in a growth chamber at 22 ± 1 C with a light intensity of 2,500 lux at bench level. The growth changer was programmed at 14 h light and 10 h darkness.

Two months after inoculation, root nodules of 0.3 by 0.5 cm in size were selected and fixed in 1% glutaraldehyde in 0.05 M cacodylate nitrate buffer, pH 7.4, containing 4.5% dextrose. The root nodules were cut in half longitudinally, while they were still in the fixative, to facilitate penetration. After fixation for 20 min, all materials were washed overnight in the same buffer before incubation for the histochemical reaction. The histochemical test was performed ac-

cording to the method described by Reik et al. (12), with some changes in conditions which will be discussed later.

The basic incubation medium consisted of 80 mM tris(hydroxymethyl)aminomethane-maleate (pH 7.4), 8% dextrose, 2 mM theophylline, 4 mM magnesium sulfate, 4.8 mM lead nitrate, and 12.5 $\times$ 10^{-3} M sodium fluoride. Treated samples were incubated in the basic medium plus 0.5 mM adenosine 5'-triphosphate (ATP). Control samples were incubated only in the basic medium. After incubation at 30 C for 1 h, the materials were washed briefly, postfixed in 1% osmium tetroxide in 0.05 M Veronal acetate buffer, pH 7.4, containing 7.5% dextrose for 4 h, and processed for electron microscopy. Thin sections were examined in the electron microscope with or without further staining. For further staining, uranyl acetate and lead citrate were used.

RESULTS

It must be noted that histochemically tested preparations are usually observed without further staining to avoid lead precipation which may be introduced in the staining processes. However, the present results indicate that staining did not alter any essential feature of the micrographs. Instead, it enhanced the overall contrast, which aided identification of the cellular organelles. For this reason, micrographs of both stained and unstained preparation are presented.

Electron-dense reaction products were found on the plasma membrane of the host cell(Fig. 1, 2, 3), and on the membrane envelopes of the bacteroids (Fig. 1, 3, arrows) but not in Golgi apparatus, mitochondrial membranes, endoplasmic reticula, vacuoles (Fig. 2, 3), or the plasma membranes of the bacteroids (Fig. 1, 3). The dense granules in the mitochondria and the bacteroids and plastids (Fig. 1, 3, 4, 5) were present, regardless of the presence of ATP in the reaction mixture, and, hence, did not appear due to adenyl cyclase. In controls, similar electron-dense products were not observed in the plasma membrane of the host cell, the membrane envelopes of the bacteroids, or membranes of other cell organelles (Fig. 4, 5).

Striking differences between the vacuoles and the membrane envelopes were observed. In sections cut through the membrane envelopes in areas where bacteroids were not present, the membrane envelopes were practically indistinguishable from vacuole membranes. However, with the histochemical test they were easily differentiated (Fig. 1).

The presence of adenyl cyclase in the plasma membrane of the host cell and the membrane envelopes of the bacteroids provides positive evidence that these two members are closely related.

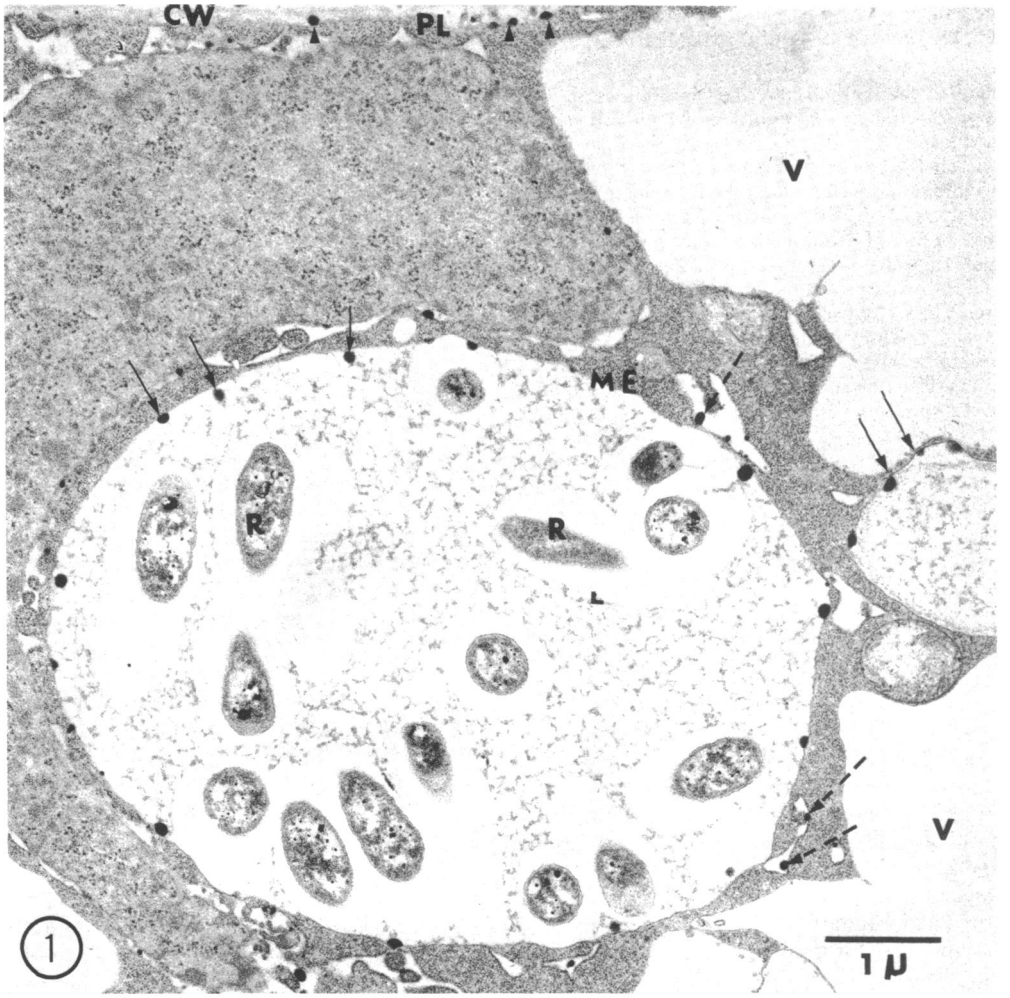
Careful examination of the micrographs showed that the dense deposits were located on the outer surface of the plasma membrane and on the inner surface of the membrane envelopes of the bacteroids (Fig. 1, 3). These results suggest that the formation of the membrane envelopes of the bacteroids was in a manner similar to endocytosis, as shown in the schematic drawing (Fig. 6). The inner surface of the membrane envelopes of the bacteroids corresponds with the outer surface of the plasma membrane. This observation was further supported by a micrograph obtained from a control sample showing an infection thread with an opened tip (lack of cell wall at the tip) which was surrounded by the plasma membrane (Fig. 5). The bacteria in the infecton thread were in the process of entering the cytoplasm endocytotically.

The results obtained in this study are in agreement with the hypothesis of Bergersen and Briggs (1), Goodchild and Bergersen (6), and Dixon (5) that, after the rhizobia are freed from the infection threads, they enter the cell endocytotically and their membrane envelopes might be related to the plasma membrane of the cell.

DISCUSSION

Cyclic adenosine 3',5'-monophosphate has been regarded as a secondary messenger, because it plays an important role in regulating cellular processes in plants and in animals (2, 11, 13, 14). Cyclic adenosine 3',5'-monosphsphate is produced through the enzymatic action of adenyl cyclase on ATP to form cyclic adenosine 3',5'-monophosphate and pyrophosphate. Since the detection of adenyl cyclase activity in this experiment has been based on lead precipitation by phosphate ions to produce the electron-dense product, lead phosphate (12), it must be pointed out that, although there are many enzymes capable of mediating the production of phosphate ions from ATP (e.g., adenosine triphosphatase, phosphotases, etc.), these phosphotases are known to be inhibited by NaF (3). On the contrary, adenyl cyclase is the only enzyme known not only to remain uninhibited but also to be stimulated by NaF. Thus, with sufficient concentration of NaF in the incubation medium, the reaction is highly specific for adenyl cyclase. This is the basis of the histochemical test of Reik et al. (12).

In this experiment, the method of Reik et al. (12) was followed closely except for some minor



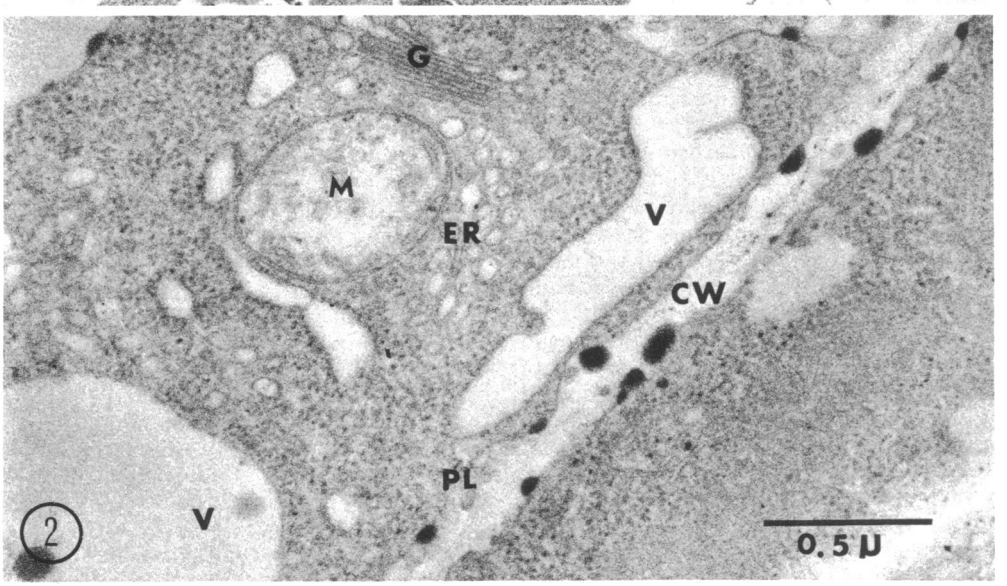


FIG. 1–6. *The abbreviations used in the figures are as follows: CW, cell wall; ER, endoplasmic reticulum; G, Golgi body; M, mitochondrion; ME, membrane envelope; ML, middle lamella; Os, osmiophilic body; Pd, plastid; PL, plasma membrane; PW, primary cell wall; R, rhizobial bacteroid; Th, infection thread; and V, vacuole.*

FIG. 1. *Stained thin section of root nodule central tissue cells. The root nodule tissue was incubated in a medium containing ATP. The reaction products are localized in the plasma membrane (arrow heads), and*

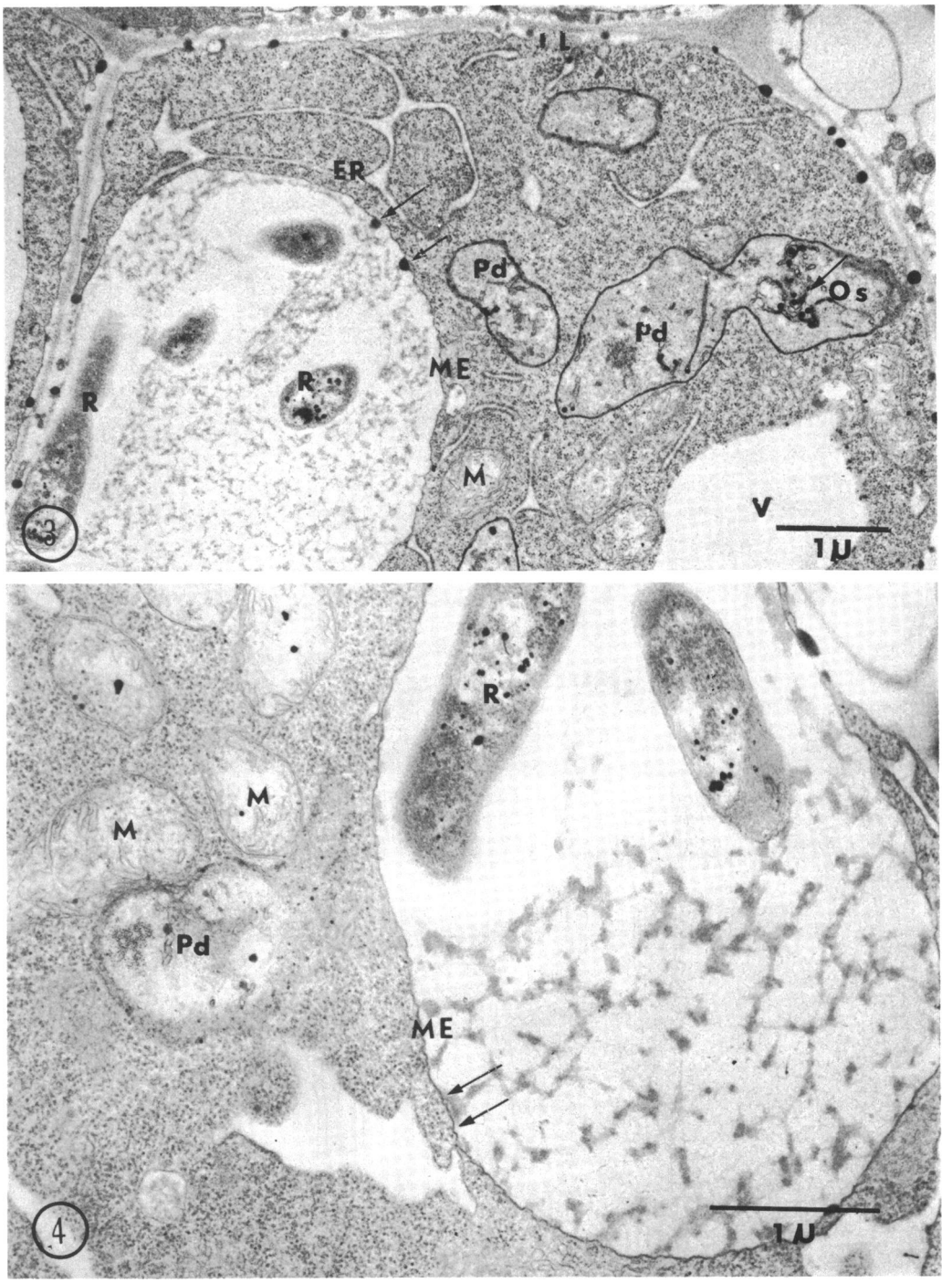


FIG. 3. *Stained thin section of root nodule central tissue cells showing the distribution of dense reaction products on the host plasma membrane and the membrane envelope (arrows) of rhizobial bacteroids, although they are absent on membranes of mitochondria, endoplasmic reticula, and vacuoles. Dense reaction products in the membrane of plastids are minimal if present. The dense granules in the plastids are osmiophilic bodies.*

FIG. 4. *Thin section obtained from root nodule incubated in the medium without ATP, showing lack of dense reaction, products on the membrane envelope of the bacteroids (arrows).*

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membrane envelopes of bacteroids (arrows) and their invaginations (broken arrows). Membranes of mitochondria, vacuoles, and the plasma membranes of the bacteroids are free of dense reaction products.

FIG. 2. *Unstained thin section obtained from material comparable to that in Fig. 1 showing Golgi body, vacuoles, endoplasmic reticula, and mitochondria free of dense deposits.*

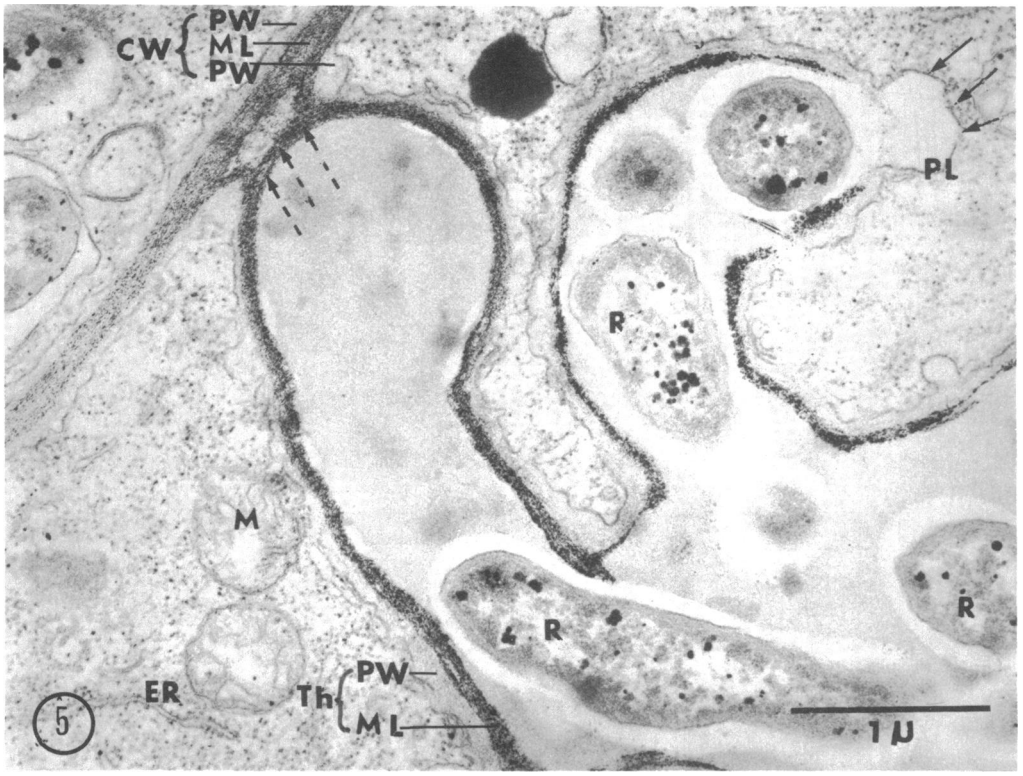

FIG. 5. *Preparation comparable to that of Fig. 4 showing that the host plasma membrane lacks electron-dense reaction products. Note: The infection thread has emerged from the middle lamella of the cell wall (broken arrows) and is open at the tip where the host plasma membrane is invaginated (arrows) to accommodate the release of a rhizobium.*

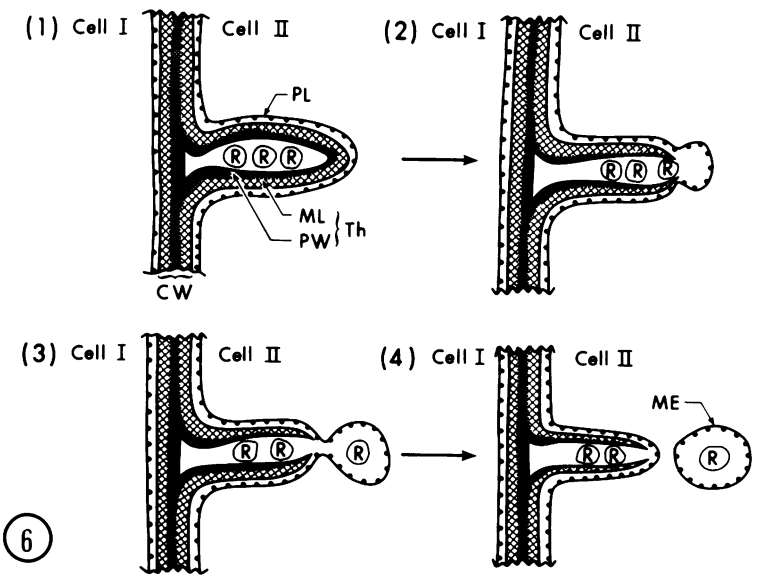

FIG. 6. *Schematic drawing to show the possible entry of rhizobia into the host cytoplasm by endocytosis, and the relationship of the membrane envelope with the host plasma membrane. Black dots indicate the location of electron-dense reaction products on the outer surface of the plasma membrane and inner surface of the membrane envelope.*

changes. Since adenyl cyclase was relatively stable at room temperature, the reaction was satisfactory when all reagents were kept at room temperature prior to preparation of the incubation medium. Freshly prepared (1 day before using) reagents proved to be most efficient. ATP was added immediately prior to incubation. After an initial equilibrium time of 10 min in a water bath at 30 C, lead nitrate was then added. Occasional shaking of the vials was necessary during incubation.

For the clover root nodule tissue, fixation of 25 to 30 min yielded satisfactory results. The same tissue, fixed 1 h or more, often failed to produce a visible histochemical reaction. This was probably due to inactivation of adenyl cyclase resulting from prolonged glutaraldehyde fixation. Experiments on rat liver showed that adenyl cyclase activity in 1% glutaraldehyde-fixed tissue varied from 10 to 50% of that in unfixed tissue (12).

As mentioned previously, there are several hypotheses on the origin of the membrane envelopes of the bacteroids. These hypotheses were based on observations made on the root nodules of various species of legumes and on preparations sectioned for routine electron microscopy. Whether the origin of the membrane envelopes of the bacteroids in various species of legume root nodules differs is not known. The present investigation shows that histochemical localization of adenyl cyclase can be used to demonstrate the relationship between the host plasma membrane and the membrane envelopes of bacteroids. My observation of the relationship between these two membranes is consistent with the hypothesis that the rhizobia may enter the host cytoplasm endocytotically and that the membrane envelope may originate from the host plasma membrane (1, 5, 7). If different species of *Rhizobium* enter the host cells by endocytosis, then other hypotheses on the biogenesis of membrane envelopes of bacteroids are disputable.

ACKNOWLEDGMENTS

The financial support from the National Research Council of Canada is acknowledged.

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