# Biogenesis of the peribacteroid membrane in root nodules

Desh Pal S. Verma and Zonglie Hong

The soil bacteria (*Rhizobium* spp.) inhabit roots of legume plants in a symbiotic association forming root nodule structures. In nodules, the bacteroids are enclosed in a membrane envelope, the peribacteroid membrane (PBM). This membrane is morphologically derived from the host plasma membrane during the infection process but undergoes rapid transformation into a specialized endosymbiotic compartment having properties in common with the vacuole. PBM biosynthesis involves unique targeting of proteins and coordination of synthesis of membrane components with bacterial cell division during early nodule development. Formation of the PBM is also controlled by the microsymbiont because mutations in *Rhizobium* genes affect the synthesis and stability of this membrane.

**An infected root nodule cell may contain several thousand rhizobial symbionts, each enclosed in a membrane envelope, the peribacteroid membrane (PBM). The PBM is derived from the host plasma membrane, but shares properties with the vacuolar membrane and contains several nodule-specific proteins (nodulins) that perform unique functions for symbiosis.**

*D.P.S. Verma* and Z. Hong are in the Dept of Molecular Genetics and Plant Biotechnology Center, Ohio State University, 1060 Carmack Road, Columbus, OH 43210, USA.*
**tel: +1 614 292 3625, fax: +1 614 292 5379, e-mail: dverma@magnus.acs.ohio-state.edu*

## Infection thread growth and formation of the PBM

Rhizobia attach to the host root hairs causing a characteristic curling, and invade the plant through a newly formed cell-wall tube, called the 'infection thread'. As the infection thread grows, host cells in the root cortex begin to divide and form the nodule primordia (Fig. 1).

The growth of the infection thread is guided by a well-organized cytoskeletal system[1], and bears some analogy with the formation of the cell plate (the new cell wall formed between daughter cells during mitosis in plants). Both the infection thread tip and cell plate grow by accumulation and fusion of Golgi-derived vesicles with plasma membrane components carrying cell-wall material[2]. During late stages of mitosis, two arrays of microtubules on either side of the cell plate form a cylindrical structure called the phragmoplast. A transient phragmoplast-like structure is also formed around the tip of the infection thread and appears to facilitate docking of the vesicles involved in infection thread growth. We have recently identified a phragmoplast-associated protein, known as phragmoplastin[3]. This dynamin-like protein may participate in the fusion of vesicles and unloading the contents required for the growing infection thread, similar to that in cell plate formation[3].

When the infection thread reaches a target cell, synthesis of infection thread wall stops and only the plasma membrane remains, which surrounds the tip of the infection thread[4]. Continuous fusion of the vesicles in this region leads to the formation of an enlarged 'unwalled droplet'. Rhizobial cells are engulfed by the plant plasma membrane, which is eventually pinched off from the droplet to form a peribacteroid unit (PBU). A single PBU may contain one to several bacteroids surrounded by PBM. As rhizobia inside the PBM continue to divide, the PBM may either divide with each bacterium or enlarge to accommodate up to eight bacteria. Both events require coordination between the deposition of membrane components to the PBM and bacterial cell division. Failure in such coordination may result in the breakdown of the PBM or in an excessively enlarged PBM. The release of rhizobia from the infection thread resembles endocytosis in eukaryotic cells.

## Composition and function of the PBM

The PBM possesses features common to both the plasma membrane and the vacuolar membrane of the host and appears to be a mosaic membrane. Both the plasma membrane and the PBM are identical in thickness (about 8–10 nm). Phosphotungstic acid (PTA) stains both the plasma membrane and the PBM but not the other endomembranes of the host cell[5]. The PBM contains two types of ATPases: $K^+/H^+$-ATPase, which is a typical plasma membrane enzyme, and $Mg^{2+}/H^+$-ATPase, which is also present in Golgi and tonoplast membranes[5,6]. The peribacteroid space (i.e. the matrix between the PBM and bacteroid) contains protease, acid trehalase, α-mannosidase II and protease inhibitors, all of which are typically found in vacuoles[7]. Compared with other endomembranes, the PBM has a characteristically high lipid:protein ratio. The most important feature of the PBM is the presence of a group of nodule-specific proteins (nodulins) that perform specialized functions unique to this membrane. Among these, nodulin-26 is the most prominent. This protein forms an active channel that is apparently regulated via phosphorylation by a protein kinase located in the PBM[8]. Homologs of this protein, with molecular masses of 26–28 kDa, are present in vacuolar and plasma membranes. These proteins belong to the major intrinsic protein (MIP) family[9].

The PBM has two major functions. First, it provides a physical barrier protecting the host cytoplasm from any direct contact with the invading prokaryote, which

Copyright © 1996 Elsevier Science Ltd. All rights reserved. 0966 842X/96/$15.00 PII: S0966-842X(96)10053-6

otherwise may interfere with the normal metabolism of the host cell and may provoke host defense responses. Second, the PBM controls the exchange of substrates and signal molecules between the two partners. The primary exchange between the two organisms is the supply of carbohydrates from the plant in return for fixed nitrogen (ammonia) from bacteroids. It is generally believed that sugars (sucrose, glucose and fructose) do not serve as the direct carbon source provided by the host because the PBM is essentially impermeable to these compounds[10]. Amino acids, such as proline and glutamate, could serve as a possible carbon source for the bacteroid[11,12]; however, no carriers for these amino acids have been found in the PBM[10]. *Rhizobium* mutants, defective in the gene for dicarboxylate transport (*dct*), are unable to take up succinate, fumarate and malate. They form nodules that contain normally differentiated bacteroids and accumulate large amounts of starch, but are defective in nitrogen fixation[13]. These results suggest that organic acids are the primary carbon source for bacteroids during symbiosis. This is supported by radiolabeling studies using $^{14}CO_2$ (Ref. 14). A dicarboxylate transporter has recently been identified in the PBM[10]. ATPase in the PBM may generate a proton gradient that drives the transport of dicarboxylates across the PBM (see Fig. 2).

Ammonia, the product of nitrogenase, has been long thought to leave the PBU by passive diffusion. However, the recent discovery of an ammonium channel in the PBM of soybean nodules suggests that fixed nitrogen may be transported as $NH_4^+$ through this channel[15]. A low pH environment in the peribacteroid space, generated by the PBM proton pump, may help the formation of $NH_4^+$ from $NH_3$ released from bacteroids. An $NH_4^+$-stimulated ATPase has been identified in the PBM[16]. The high $NH_4^+$ concentration in the peribacteroid space and the low concentration of $NH_4^+$ in the host cytoplasm, together with the cation gradient generated by the proton pump, may create a force driving $NH_4^+$ through the 'PBM-$NH_4^+$ channel'. Compared with the high-affinity $NH_4^+$ transporter ($K_m$ of 10 μM), recently identified in *Arabidopsis*[17], the PBM–$NH_4^+$ channel has a much lower affinity for ammonium ($K_m$ of 37.5 mM). However, since $NH_4^+$-channel density is high on the PBM, it can easily accommodate physiological rates of $NH_4^+$ uptake into the host cell. The PBM allows transport of inorganic minerals (e.g. iron and sulfur) to the bacteroid and export of heme from the bacteroid to the plant cytoplasm for leghemoglobin synthesis[18]. Specific transporters may be involved in these processes.

## PBM biogenesis

The biogenesis of PBM imposes several new problems because of its mosaic nature. To acquire new function several new membrane components, including lipids and membrane proteins, are incorporated into this membrane. Some of the new lipids in this membrane may play a role in determining vesicle docking sites. A new glycolipid, belonging to a family of molecules not previously identified in plants, has been observed in the PBM by Brewin's group using specific antibodies[19].

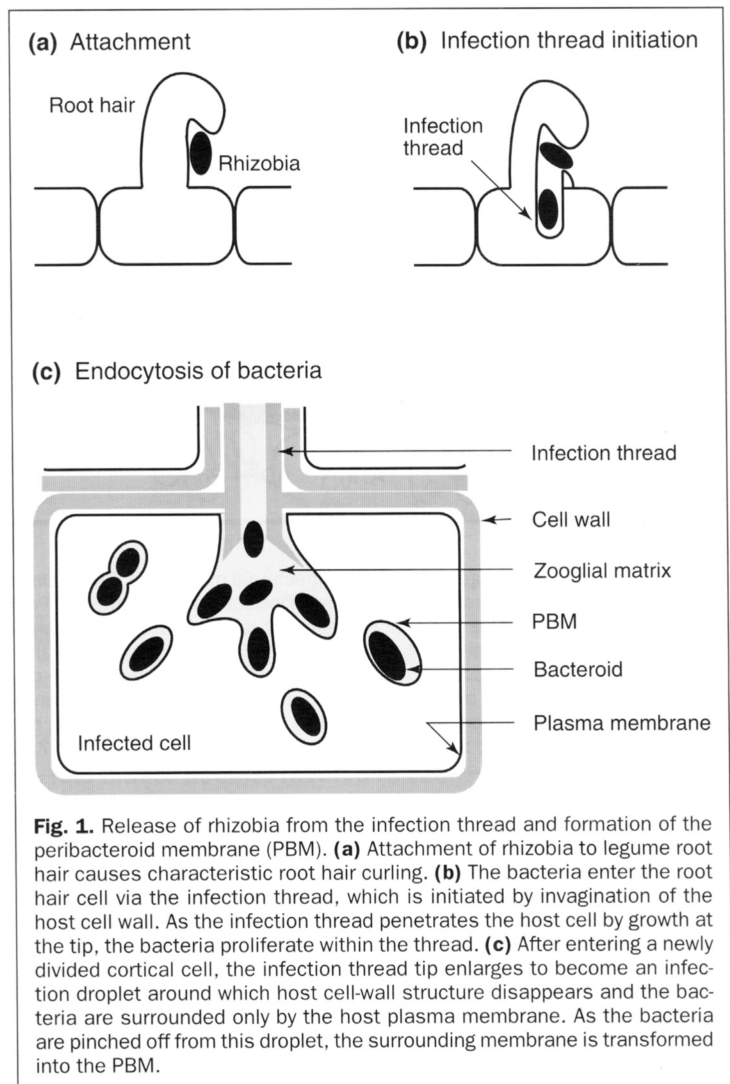


**Fig. 1.** Release of rhizobia from the infection thread and formation of the peribacteroid membrane (PBM). **(a)** Attachment of rhizobia to legume root hair causes characteristic root hair curling. **(b)** The bacteria enter the root hair cell via the infection thread, which is initiated by invagination of the host cell wall. As the infection thread penetrates the host cell by growth at the tip, the bacteria proliferate within the thread. **(c)** After entering a newly divided cortical cell, the infection thread tip enlarges to become an infection droplet around which host cell-wall structure disappears and the bacteria are surrounded only by the host plasma membrane. As the bacteria are pinched off from this droplet, the surrounding membrane is transformed into the PBM.

In animals, such lipids are thought to be cell recognition factors and are involved in many bacterial infections. A new isoform of choline kinase and phosphatidylinositol (PI) 3-kinase have also been detected in nodules[20,21]. The latter is of particular interest because PI 3-kinase is essential in protein targeting to the vacuole in yeasts and has been shown to participate in PBM proliferation in root nodules[21]. Newly synthesized lipids and proteins are transported to the PBM via the vesicles derived from the Golgi. Targeting of PBM-specific proteins appears to follow different routes (see below), and no conserved targeting mechanism has yet emerged.

### *Vesicular traffic and small GTP-binding proteins*

Vesicle budding and fusion are active in the proximity of the PBM and the growing infection threads. Both smooth and coated vesicles have been found to associate with the PBM and growing infection threads in the infected root nodule cells[22–24]. Coated vesicles associated with PBM are generally larger in size than those associated with plasma membrane[22]; clathrin-coated vesicles have been isolated from plants and found to participate in endocytosis[25]. Although the exact role of smooth and coated vesicles in PBM biogenesis remains

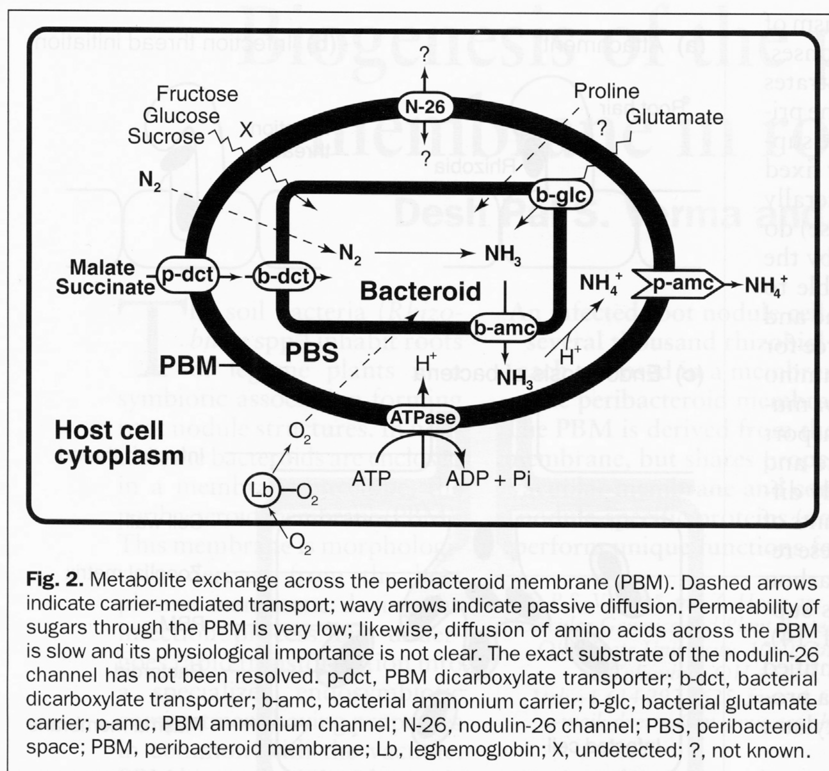


**Fig. 2.** Metabolite exchange across the peribacteroid membrane (PBM). Dashed arrows indicate carrier-mediated transport; wavy arrows indicate passive diffusion. Permeability of sugars through the PBM is very low; likewise, diffusion of amino acids across the PBM is slow and its physiological importance is not clear. The exact substrate of the nodulin-26 channel has not been resolved. p-dct, PBM dicarboxylate transporter; b-dct, bacterial dicarboxylate transporter; b-amc, bacterial ammonium carrier; b-glc, bacterial glutamate carrier; p-amc, PBM ammonium channel; N-26, nodulin-26 channel; PBS, peribacteroid space; PBM, peribacteroid membrane; Lb, leghemoglobin; X, undetected; ?, not known.

unknown, it is possible that the deposition of membrane proteins to the PBM and soluble proteins to the peribacteroid space could employ both smooth and coatomer-coated vesicles, whereas clathrin-coated vesicles may be involved in the reception of signal molecules from the bacteroids[26] (Fig. 3).

The PBM must contain unique receptors that are recognized by the Golgi-derived vesicles but such receptors are not expected to be present in the plasma membrane or vacuolar membranes. A family of small GTP-binding proteins (mainly Rab proteins) have been shown to be involved in the regulation of membrane traffic in eukaryotic cells[27]. Several Rab homologs have been identified in root nodules. Expression of the *rab7* gene is enhanced significantly during nodulation, and inhibition of *rab7* expression in soybean nodules has been found to result in the accumulation of late endosomes and multivesicular bodies in the perinuclear region[28]. This study also suggested that endocytosed vesicles containing bacteria may undergo a maturation phase and that retardation of vesicle fusion by antisense *Rab7* transforms these vesicles into a lytic compartment where bacteria are killed. However, similar experiments with antisense *Rab1* blocked membrane flow and resulted in a breakdown of the PBM. Another small GTP-binding protein, Sar1, has been shown to be essential for vesicle budding from the endoplasmic reticulum (ER) in yeast[29]. Four *Sar1* homologous cDNAs have been cloned from soybean, and although the *Sar1* homologs are expressed in all plant tissues examined so far, one is specifically induced in nodule tissue (Z. Hong and D.P.S. Verma, unpublished). These data suggest that the level of small GTP-binding proteins involved in vesicular traffic is enhanced during nodule formation to control vesicle flow for PBM biogenesis. Moreover, the PBM contains unique small-GTP binding proteins that could play roles in PBM proliferation during nodule formation (D.P.S. Verma and Z. Hong, unpublished).

*Targeting of nodulins to the PBM*

Since the amount of PBM is 20–40-fold greater than that of plasma membrane in an infected cell, most of the newly synthesized membrane proteins in the infected cells must be targeted to the PBM. In plant cells, the targeting of proteins from the Golgi body to the plasma membrane does not require a specific signal sequence. In contrast, tonoplast proteins generally contain vacuolar targeting sequences that ensure the delivery of these proteins to the vacuolar membrane[30]. Unlike nuclear, plastidic, mitochondrial and peroxisomal proteins, which are synthesized on free ribosomes and post-translationally imported into these organelles, PBM proteins have been shown to be synthesized on membrane-bound ribosomes and cotranslationally inserted into the ER[31]. These proteins are processed in the Golgi body, packaged into the vesicles and delivered to the PBM.

It is clear that protein targeting to the PBM does not follow the default pathway because PBM nodulins (like nodulin-24 and nodulin-26) are not found in the plasma membrane. Specific targeting information must be carried in these nodulins. Analysis of nodulin-23, nodulin-24 and nodulin-26 has not revealed any conserved features, neither at the primary nor at the secondary structure levels[32]. Nodulin-26 shares significant sequence homology with the MIP family, in that these proteins consist of six putative membrane-spanning domains and form channels in membranes. The *Escherichia coli* MIP, GlpF and the yeast MIP, Fps1, facilitate the translocation of glycerol and other small molecules across the membranes. The plant (γ-TIP) and human (CHIP28) members form water channels (known as aquaporins), which allow water to pass freely while simultaneously excluding ions and metabolites. While the nature of the substrate that passes through the nodulin-26 channel remains unknown, it has been suggested that this channel is not an aquaporin and either may be responsible for the translocation of host metabolites (presumably dicarboxylates, such as succinate and malate) to the bacteroids, or may act as an ammonium channel[8,9]. When expressed in transgenic tobacco, nodulin-26 is targeted to the vacuole membrane, which confirms that the vacuoles have properties in common with the PBM, and in the absence of PBM it can serve as a target membrane for PBM nodulins[33].

Newly synthesized nodulin-24 is processed cotranslationally by the removal of the amino-terminal signal sequence and is released as a 20-kDa polypeptide into the ER lumen. Further post-translational processing (presumably in the Golgi) attaches this protein to the membrane, resulting in the increase of its apparent molecular mass to 33 kDa. This protein does not have

any transmembrane domain but contains a characteristic lipid-binding motif. The latter may allow this nodulin to be buried in the lipids of the PBM surface facing the bacteroids[34]. Although the function of nodulin-24 is unknown, the amphipathic nature of this protein may afford a recognition site through which bacteroids may attach themselves to the PBM surface. Direct contact between the bacteroid and the PBM may be important for the coordination of PBM growth with rhizobial cell division during early stages of infection.

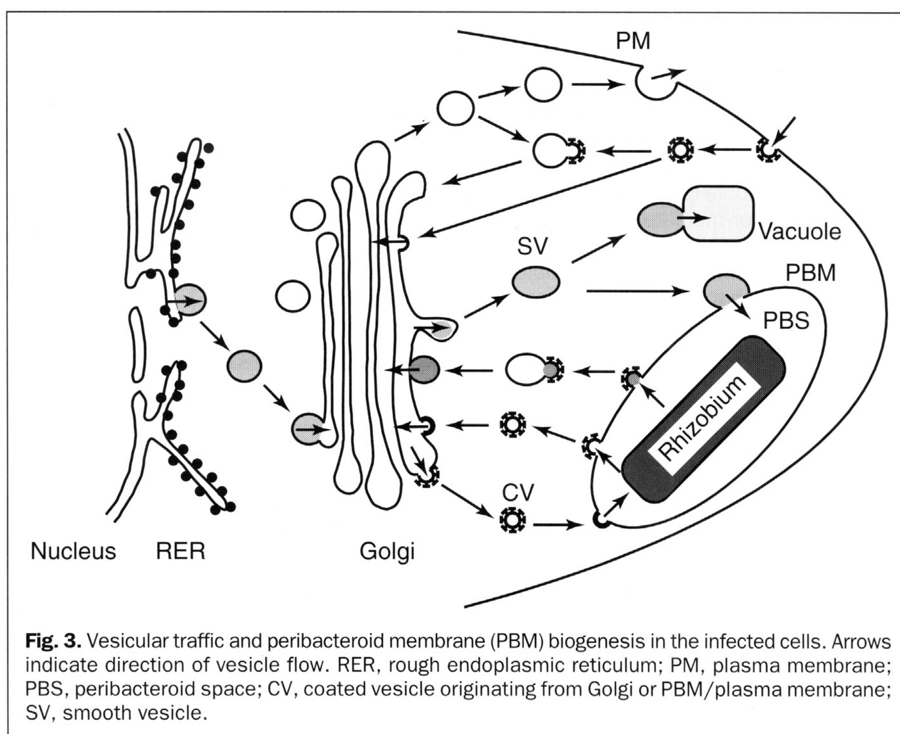


**Fig. 3.** Vesicular traffic and peribacteroid membrane (PBM) biogenesis in the infected cells. Arrows indicate direction of vesicle flow. RER, rough endoplasmic reticulum; PM, plasma membrane; PBS, peribacteroid space; CV, coated vesicle originating from Golgi or PBM/plasma membrane; SV, smooth vesicle.

### Contribution of *Rhizobium* to PBM synthesis

The establishment of successful symbiosis and the formation of functional PBM requires the expression of numerous bacterial and plant genes. The microsymbiont may contribute to PBM biogenesis in two ways: by direct provision of PBM proteins and by secretion of yet unidentified signal molecules that control host vesicle flow to the PBM. A 31-kDa protein has been found that crossreacts with anti-PBM, anti-PBS and anti-bacteroid antisera[35]. The possibility exists that this protein is synthesized and secreted by bacteroids. The 31-kDa protein has not been found in the PBM or in the peribacteroid space of nodules induced by *R. japonicum* mutant SM5, which contain normal size PBUs but are defective in nitrogen fixation[36]. It is not clear whether the mutated rhizobial gene directly encodes the 31-kDa protein or whether the gene product somehow acts as a signal controlling the expression of this protein.

Another group of *Rhizobium* mutants induce nodules that produce 'empty' PBMs devoid of bacteroids. In these nodules, expression of nodulin-26 is not affected, while nodulin-24 mRNA is strongly depressed[36]. Only four PBM nodulins are present in empty PBM, whereas PBM from wild type nodules contain at least seven major nodulins[37]. The expression of some PBM nodulins does not seem to be dependent on the presence of rhizobia within the PBM and their expression is induced as soon as nodule organogenesis is committed. Other PBM nodulins are induced only when rhizobia are properly endocytosed. The fact that a mutation in a rhizobial processing protease can affect PBM biogenesis suggests a direct involvement of some bacterial proteins in this process[38].

### Unresolved questions

Significant progress has been made in investigating the synthesis and biological function of the rhizobial Nod factors that are capable of making nodule-like structures in the absence of bacteria[39]. Nod factors, however, do not appear to participate in PBM proliferation, because many *Rhizobium* mutants that apparently synthesize and secrete normal amounts of Nod factors induce nodules that fail to endocytose or that cannot synthesize functional PBM. There seems to be another group of rhizobial signals whose primary function is to trigger endocytosis in the host, including the release of rhizobia from the infection threads followed by internalization of the bacteria. To differentiate these signals from Nod factors, we call them Edo factors, for endocytosis factors. Some *Rhizobium* Bar$^-$ (bacteria release) and Fix$^-$ (nitrogen fixation) mutants affecting endocytosis can be included in this group. Further identification of new *edo* loci may eventually uncover the nature of Edo factors. Such bacterially secreted molecules may interact with specific receptors on the PBM or may pass through PBM via coated vesicles. One of the endocytosis-defective mutants, T8-1 (Ref. 36), has recently been shown to fail to synthesize cytochrome *c* (Ref. 40, and H. Hennecke, pers. commun.). It is not known how cytochrome *c* synthesis in rhizobia is related to their endocytosis, and perhaps it simply arrests the growth of bacteria in the infection thread.

Proliferation of PBM requires massive vesicle fusion with this membrane. How do vesicles carrying PBM nodulins recognize PBM but not plasma or vacuolar membranes? Based on the model of vesicle fusion generated from mammalian studies, specific recognition is achieved by the interaction between a receptor on the donor vesicle and a receptor on the target membrane[26]. Such receptors for PBM biogenesis must be in some way different from those of the plasma membrane and the vacuolar membrane, and clearly warrant further investigation.

Since nodules are not essential to the life of a plant, and various mutations can be created experimentally, the root nodule is an ideal system for studying vesicle budding and fusion and membrane biogenesis in plants. The cloning of genes encoding proteins that participate

> **Questions for future research**
>
> - The peribacteroid membrane (PBM) is derived from the host plasma membrane but shares properties with the vacuolar membrane. How is the transformation from plasma membrane to PBM achieved? How does the PBM gain the properties of a vacuolar membrane?
> - How are rhizobia released from the infection threads? Do they follow a receptor-mediated endocytosis process or integrin-mediated phagocytosis?
> - How are PBM nodulins (e.g. nodulin-24 and nodulin-26) targeted to the PBM, but not to the plasma or vacuolar membrane?
> - How do rhizobia affect PBM biosynthesis and stability? Are there PBM proteins that are encoded by rhizobia? If yes, how are they integrated into the PBM?
> - How is PBM synthesis coordinated with rhizobial cell division at the early stages of infection?

in and control these processes is a major but achievable task in the light of recent progress made in membrane biogenesis studies of yeast and mammalian systems. Eventually, development of an *in vitro* vesicle fusion system will be essential to decipher the detailed reactions involved in specific steps of vesicle traffic and PBM biogenesis.

### References


1 van Brussel, A. *et al.* (1992) *Science* 257, 70–72
2 Brewin, N. (1991) *Annu. Rev. Cell Biol.* 7, 191–226
3 Gu, X. and Verma, D.P.S. (1996) *EMBO J.* 15, 695–704
4 Newcomb, W. (1976) *Can. J. Bot.* 54, 2163–2186
5 Verma, D.P.S. *et al.* (1978) *J. Cell Biol.* 78, 919–936
6 Bassarab, B., Mellor, R.B. and Werner, D. (1986) *Endocyt. Cell Res.* 3, 189–196
7 Mellor, R. (1989) *J. Exp. Bot.* 40, 831–839
8 Miao, G-H., Hong, Z. and Verma, D.P.S. (1992) *J. Cell Biol.* 118, 481–490
9 Verma, D.P.S. (1992) in *Transport and Receptor Proteins of Plant Membranes* (Cook, D.T. and Clarkson, D.T., eds), pp. 113–117, Plenum Press
10 Udvardi, M.K. *et al.* (1990) *Mol. Plant–Microbe Interact.* 3, 334–340
11 Kahn, M.L., Kraus, J. and Somerville, J.E. (1985) in *Nitrogen Fixation Research Progress* (Evans, H.J., Bottomley, P.J. and Newton, W.E., eds), pp. 193–199, Martinus Nijhoff
12 Kohl, D. *et al.* (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 2036–2040
13 Ronson, C.W., Lyttleton, P. and Robertson, J.G. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 4284–4288
14 Salminen, S. and Streeter, J. (1992) *Plant Physiol.* 100, 597–604
15 Tyerman, S., Whitehead, L. and Day, D. (1995) *Nature* 378, 629–632
16 Blumwald, E. *et al.* (1985) *Plant Physiol.* 78, 665–672
17 Ninnemann, O., Jauniaux, J. and Frommer, W. (1994) *EMBO J.* 13, 3464–3471
18 McGinnis, S.D. and O'Brian, M.R. (1995) *Plant Physiol.* 108, 1547–1552
19 Perotto, S. *et al.* (1995) *Mol. Plant–Microbe Interact.* 8, 560–568
20 Mellor, R., Christensen, T. and Werner, D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 659–663
21 Hong, Z. and Verma, D.P.S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 9617–9621
22 Robertson, J. and Lyttleton, P. (1982) *J. Cell Sci.* 58, 63–78
23 Robertson, J. *et al.* (1978) *J. Cell Sci.* 30, 129–149
24 Robertson, J. *et al.* (1978) *J. Cell Sci.* 30, 151–174
25 Holstein, S.E., Drucker, M. and Robinson, D.G. (1994) *J. Cell Sci.* 107, 945–953
26 Mellor, R. and Werner, D. (1987) *Symbiosis* 3, 75–100
27 Verma, D.P.S., Cheon, C-I. and Hong, Z. (1994) *Plant Physiol.* 106, 1–6
28 Cheon, C-I. *et al.* (1993) *EMBO J.* 12, 4125–4135
29 Barlwe, C. and Schekman, R. (1993) *Nature* 365, 347–349
30 Chrispeels, M. (1991) *Plant Physiol.* 42, 21–53
31 Katinakis, P. and Verma, D.P.S. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 4157–4161
32 Fortin, M.G., Morrison, N.A. and Verma, D.P.S. (1987) *Nucleic Acids Res.* 15, 813–824
33 Zhang, Y. and Roberts, D.M. (1995) *Mol. Biol. Cell* 6, 109–117
34 Cheon, C-I., Hong, Z. and Verma, D.P.S. (1994) *J. Biol. Chem.* 269, 6598–6602
35 Fortin, M.G., Zelechowska, M. and Verma, D.P.S. (1985) *EMBO J.* 4, 3041–3046
36 Morrison, N. and Verma, D.P.S. (1987) *Plant Mol. Biol.* 9, 185–196
37 Mellor, R., Garbers, C. and Werner, D. (1989) *Plant Mol. Biol.* 123, 307–315
38 Werner, D. *et al.* (1995) in *Nitrogen Fixation: Fundamentals and Applications* (Tikhonovich, I. *et al.*, eds), pp. 449–453, Kluwer Academic Publishers
39 Verma, D.P.S. (1992) *Plant Cell* 4, 373–382
40 Ritz, D. and Hennecke, H. (1993) *Mol. Microbiol.* 9, 729–740



## The 1997 First Electronic Conference in Cell Biology

## Call for organizers

This month's issue of *trends in* CELL BIOLOGY contains an interview with Barry Hardy on virtual conferences. In this interview, Dr Hardy issues an invitation to scientists working in cell biology and related disciplines to become involved in the 1997 First International Cell Biology Electronic Conference. This is a new venture and requires a cell biological advisory committee to assist with organizing the meeting sessions and inviting presenters. Anyone interested in participating, at any level, should contact either Barry Hardy (barry@bellatrix.pcl.ox.ac.uk) or the *trends in* CELL BIOLOGY editorial team (at tcb@elsevier.co.uk or the editorial address listed at the front of this journal).