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Phil. Trans. R. Soc. Lond. B 1995 **347**, 181-185
doi: 10.1098/rstb.1995.0020

Bacterial symbionts in the epidermis of an Antarctic neopilinid limpet (Mollusca, Monoplacophora)

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SUMMARY

For the first time it has become possible to study a 'living fossil' *Laevipilina antarctica*, a representative of the family Neopilinidae (Mollusca, Monoplacophora) by means of transmission electron microscopy. This led to the discovery of a bacterial symbiosis in the epidermis of the mantle roof and of the head of the animal. Bacteria with varying morphologies were found between the microvilli of the epidermal cells. In addition, modified and specialized epidermal cells (bacteriocytes) were detected in the mantle roof and the post-oral tentacles. In contrast, the sole of the foot and the alimentary tract of the animal are free of symbionts. The bacterial symbionts may be involved in the recycling of dissolved organic matter.

1. INTRODUCTION, MATERIAL AND METHODS

The phylogenetic significance of recent Monoplacophora (Neopilinidae) has been accepted by all authorities. However, detailed anatomical and histological observations on these 'living fossils' have up to now been restricted to two of about 20 species described (Lemche & Wingstrand 1959; Wingstrand 1985; Warén & Hain 1992). In addition, the ultrastructure of any neopilinid is completely unknown, as is their ontogeny.

Two new neopilinid species were recently collected by the R/V *Polarstern* in the Weddell Sea (Antarctica) between 210 m and 650 m and have been described as *Laevipilina antarctica* and *Micropilina arntzi* (Warén & Hain 1992). Fortunately a single specimen (of 12) of *Laevipilina antarctica* was detected alive and preserved in 2.5% glutaraldehyde (buffered in 0.1 m cacodylate-buffer) by one of us (S.H.) providing the first opportunity to study an extant monoplacophoran species by transmission electron microscopy (TEM). After several weeks of storage in the primary fixative further treatment of this specimen was carried out in Innsbruck. After postfixation by 2% buffered osmium tetroxide and dehydration in the alcohol series the specimen was embedded in Spurr's (1969) resin. Alternate semi-thin (0.5 μm) and ultrathin (70 nm) sections were made with diamond knives, resulting in a nearly complete section series available for light microscopy as well as in short series of seven ultrathin sections each of regular distances of 10 μm. The ultrathin sections were stained by uranyl-acetate and lead-citrate after Daddow (1983), the sections were examined with a ZEISS EM902 in Innsbruck.

The microanatomy and fine-structure of *Laevipilina antarctica* will be published elsewhere (Haszprunar & Schaefer 1995). Here we report on the bacterial symbiosis in the epidermis of this species.

2. RESULTS

(*a*) *General appearance*

The whole ventral epithelium of the animal's body is covered by a microvillar border (figures 1, 9 and 10). The microvilli are circular in cross sections (diameter about 100 nm), usually branched and show a distal web of fine threads (figures 2 and 3: arrowheads), which probably is a so-called glycocalyx (see Rieger (1984) for definition). Below the glycocalyx several types of bacteria may be found in great numbers between the microvilli. The colonized area includes the whole lateral pallial roof, the head, the proximal part of the ctenidia, the lateral wall of the foot (so-called epipodium), and in particular the postoral tentacles, where the highest bacterial density occurs. In contrast, bacteria are lacking in the distal part of the ctenidial digits, in the foot sole and in the whole alimentary tract. The borders between colonized and non-colonized areas are in general well defined, there being no transitional areas. The microvillar border of ciliated cells may be colonized too. Occasionally bacteria are found in the most distal portion of regular epidermal cells suggesting phagocytosis (figure 10).

(*b*) *Bacterial types*

The majority of bacteria belong to a single 'smooth' type within which two morphs can be distinguished. The more common morph (figures 1 and 2) is of elongate shape, maximally 2.3 μm long and has a diameter of about 0.3 μm. The cytoplasm is electron-lucent with some fine threads in it, the wall is smooth consisting of homogeneous electron-dense material. The second morph (figures 7 and 9) is less common and is smaller than the first one with a maximum length of 1.8 μm and a maximum diameter of 0.2 μm. The most significant distinguishing feature is a core of electron-dense material in the cytoplasm. However, there are

Phil. Trans. R. Soc. Lond. B (1995) **347**, 181–185
Printed in Great Britain

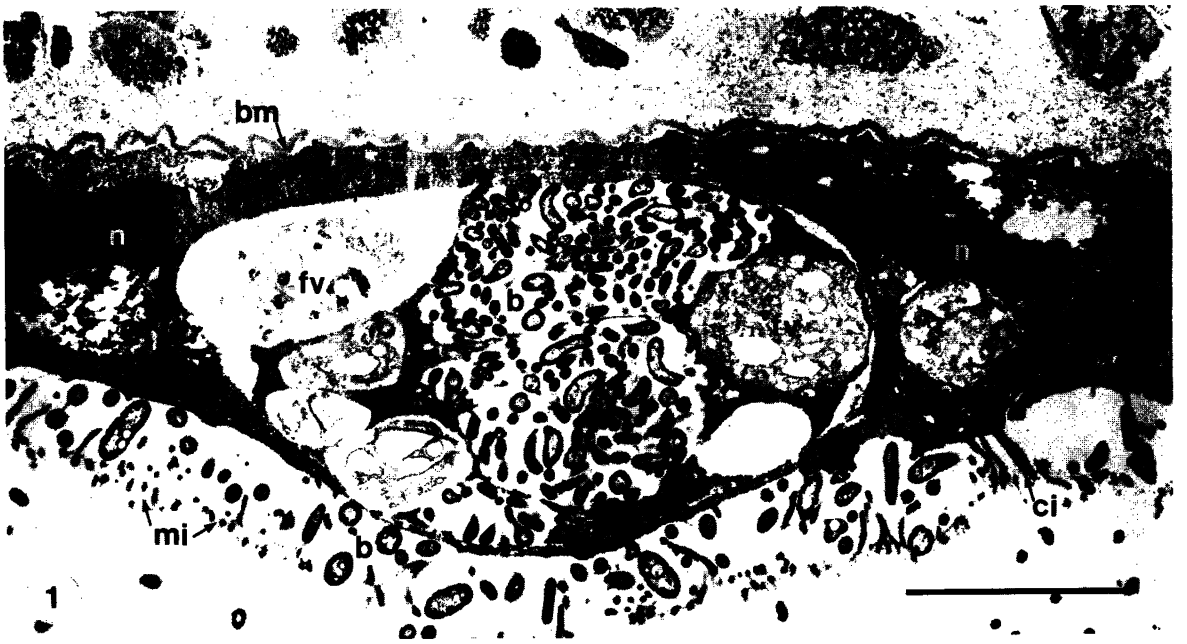


Figure 1. Low power TEM-photograph of epithelium of the mantle cavity of *Laevipilina antarctica* showing bacteria of 'smooth' type (b) in the microvillar border and within a bacteriocyte. Abbreviations: b, bacteria; bm, basement membrane; ci, cilium; fv, food vacuole; m, muscle fibre; mi, microvilli; n, nucleus. Scale bar 5 μm.

also intermediate forms between the two morphs which we regard as different stages of the life cycle with more or less condensed chromatin.

A second type of bacterium is predominately found in the anterior part of the body and, particularly in the epithelium of the postoral tentacles. It was not found in the bacteriocytes (see below) but occurs solely between the microvilli. This 'hairy' type (figure 3) shows fine threads at its surface and two distinct external membranes. In longitudinal sections three distinct zones can be distinguished, an electron-dense core being composed of fine granules, whereas towards both ends there are areas of electronlucent material. We never found intermediate forms between this and the 'smooth' type.

A further type of bacterium ('round') shows quite a different morphology and structure (figure 4 and 5). This type is found only occasionally near the distal surface of the epidermal epithelium and forms clusters in the colonized area. The nearly spherical bacterium has a diameter of nearly 1 μm and the wall is very thin compared with the other types. The chromatin is situated excentrically, the remaining volume filled with very small granules which show a central electron-dense spot.

Finally, an 'irregular' type of bacterium is infrequently present (figure 6). It is characterized by an irregular wall consisting of three layers over about three quarters of the cell surface, and a single layer over the remaining quarter. The chromatin is quite condensed in the centre of the bacterial cell.

(*c*) *Bacteriocytes*

In the colonized areas two epidermal cell types are present showing bacteria below the surface of the epithelium. The more common type differs from regular epidermal cells in exhibiting a distinct lumen which forms an elongated curved tube being connected with the epidermal surface via a narrow porus. Both morphs of 'smooth' bacteria are found between the few but distinct microvilli of the lumen (figures 1 and 7). The nucleus of the cell, which has a depressed shape, is always found adjacent to the lumen. The bacteriocyte bears few cilia at the surface of the epithelium, whereas cilia are lacking in the lumen. Very often additional large vacuoles are present in the vicinity of the lumen. These vacuoles usually include residual material, sometimes degraded bacteria are also present (figures 7 and 8).

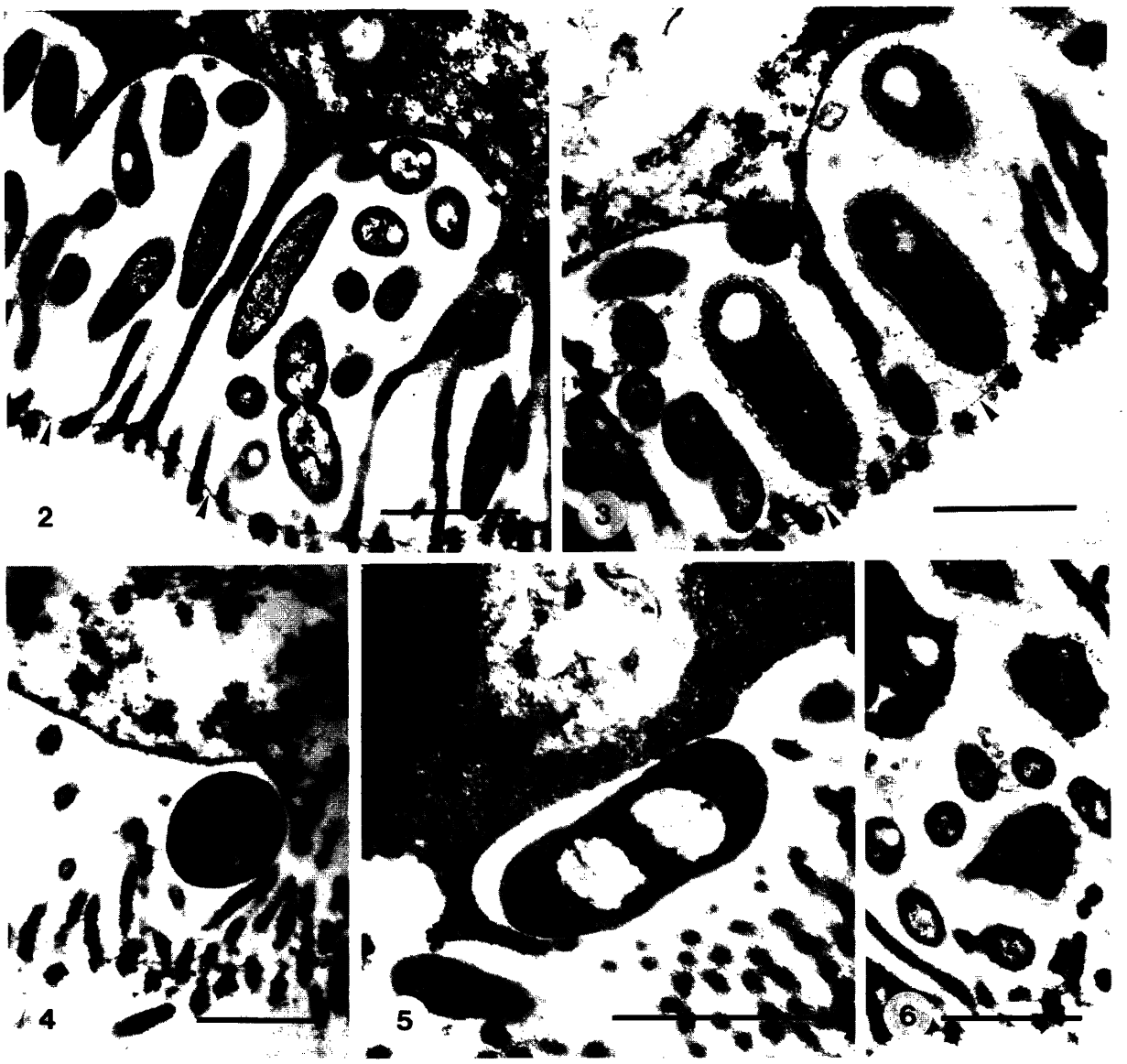
The second type of bacteriocyte (figure 9) possesses a large vacuole filled with amorphous material. Bacteria of the second morph (small, dark core) are densely packed around a central lumen where microvilli are absent. Some bacteria are embedded in the amorphous mass. Despite extensive searching a direct connection between the lumen and the epithelial surface could not be detected. This cell type lacks cilia, but exhibits a microvillar border.

3. DISCUSSION

(*a*) *Cytological aspects*

The association between *Laevipilina antarctica* and its epidermal bacteria does not seem in any way pathological. The epidermal bacteria described herein were never found in association with epidermal lesions, which were present at the posterior foot sole of the animal. Probably pathological bacteria have been found in the distal part of connective tissue, where they appear to have invaded the wounded areas and were attacked by hemocytes (phagocytes; cf. Haszprunar & Schaefer 1995).

The role of the bacteriocytes is far from clear and their specific modifications might be genuine or



Figures 2–6. Bacterial types between branched microvilli, which are distally interconnected by the glycocalyx (arrowheads). All scale bars 1 μm.

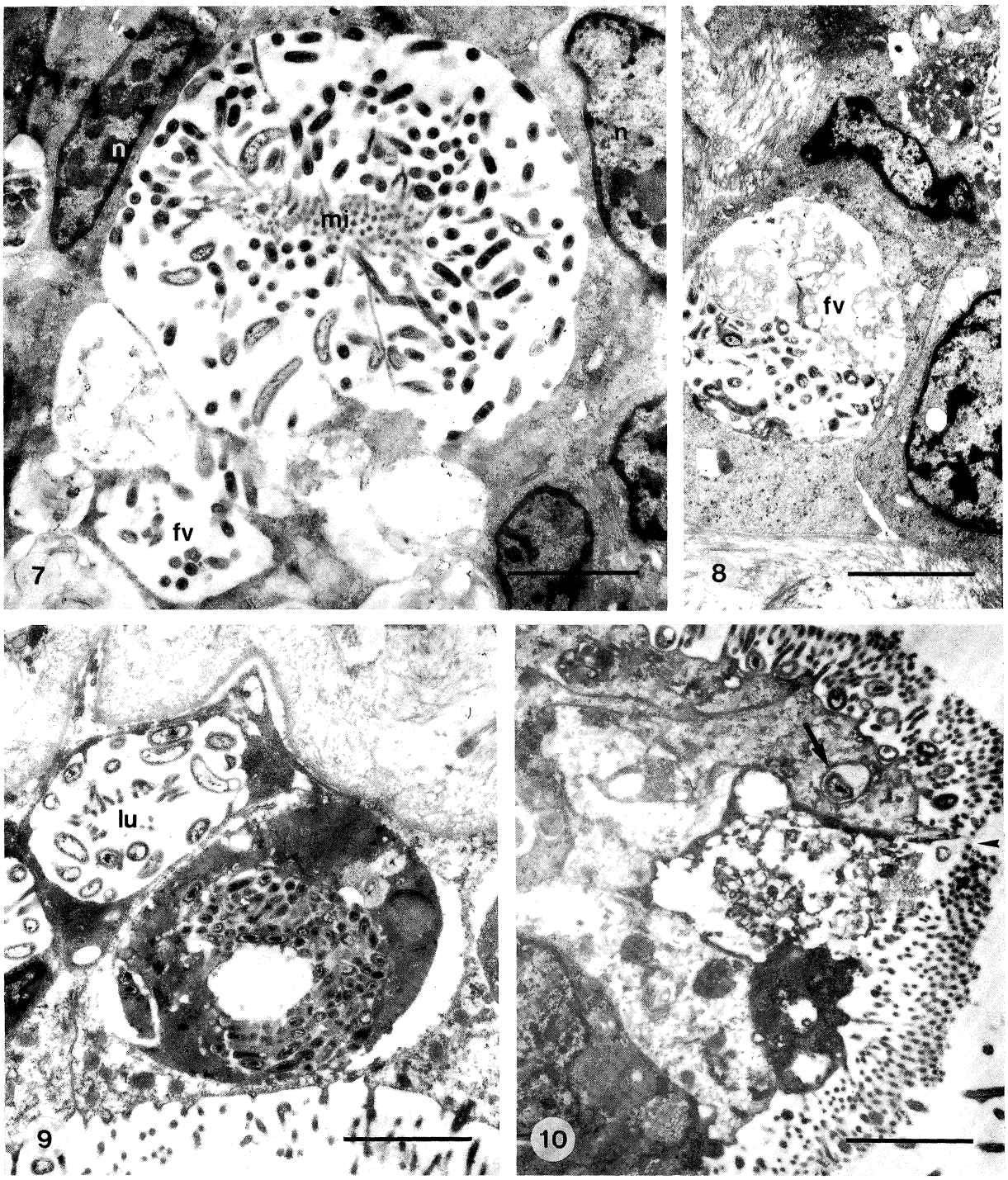
Figure 2. 'Smooth type'.
Figure 3. 'Hairy' type.
Figure 4. 'Round' type.
Figure 5. 'Round' type in division.
Figure 6. 'Irregular' type.

induced by bacterial colonization. We regard the latter possibility as less likely, because bacteriocytes are not found in all regions of the animal's body. Bacteriocytes probably feed on the bacteria, being suggested by the mixture of organic residues and more or less intact bacteria in the adjacent vacuoles (figures 1, 7 and 8).

(*b*) *Comparative aspects*

Among the aplacophoran molluscs a similar association between bacteria and pallial epidermal cells has been found in the solenogaster *Neomenia carinata* (see Scheltema *et al.* 1994) where evidence of phagocytosis also exists. Up to now there are no reports about comparable epidermal symbiosis with bacteria in polyplacophorans or gastropods, whereas many data exist for bivalves (see review in Prieur *et al.* 1990).

Symbioses of invertebrates with chemoautotrophic or heterotrophic bacteria may be classified into four major groups, although transitions are known to occur between each of them (see, for example, Ott *et al.* 1982): A first group consists of bacteria capable of bioluminescence, a possibility of little likelihood in the case of *Laevipilina*. A second group comprises animals using bacteria for the digestion of substrates such as wood which cannot be utilized without microbial activity. However, the detritovorous *Laevipilina antarctica* does not show bacteria in its gut. A third group includes all those symbiotic associations which can use sulphide as an energy source. In many such cases the animals may actually feed on the bacteria and there is an associated reduction of the alimentary tract of the host (see recent review by Saffo 1992). Examples of hosts with unmodified guts have been found among



Figures 7–10. TEM photographs of bacteriocytes of *Laevipilina antarctica*. Abbreviations: fv, food vacuole; lu, lumen; mi, microvilli; n, nucleus. All scale bars 2 μm.

Figure 7. Horizontal section of bacteriocyte of type one with food vacuoles. Note the two morphs of 'smooth' bacteria in the lumen which is bordered by microvilli.

Figure 8. Food vacuole of a type 1 bacteriocyte with degrading bacteria.

Figure 9. Bacteriocytes with bacteria. Type 1 bacteriocyte (with lumen) contains bacteria of bright morph. Type 2 bacteriocyte contains 'smooth' bacteria of dark-core morph.

Figure 10. Strict border (arrowhead) of colonized area (above) at lateral foot showing phagocytosing (arrow) epidermal cell.

gastropods from hydrothermal vents (see De Burgh & Singla 1984; Stein *et al.* 1988) or in nematodes (Ott *et al.* 1982). In the echiuran worm *Urechis caupo* chemoautotrophic bacteria appear to occur in the cuticle and are ingested by epidermal cells (Menon & Arp 1993). *Urechis caupo* is tolerant against high concentrations of hydrogen sulphide and one might also speculate about a chemoautotrophic role of the bacteria in *Laevipilina antarctica*. However, the contents of the dredgings, where this species was found, suggests an environment lacking free sulphide (Warén & Hain 1992). In addition, the symbiotic bacteria of gastropod

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and bivalves from the hydrothermal vent habitat are generally found in the ctenidial leaflets, whereas the ctenidia are free of symbionts in *Laevipilina antarctica*.

Finally, there remain cases where the hosts live in an oxygenated environment lacking free sulphide and the function of the microbial symbionts is not well defined. Such examples are found among sponges (Saffo 1992) in the enigmatic worm *Jennaria pulchra* (Rieger & Rieger 1991), among molluscs (see above), or among echinoderms (Holland & Nealson 1978; McKenzie & Kelly 1994). As in *Laevipilina antarctica* the echinoderm bacteria are situated in the microvillar border of the epidermal cells below the glycocalyx layer, but there are no bacteriocytes in the echinoderm hosts.

A more detailed discussion about the biological role of the bacteria in *Laevipilina antarctica* is hampered by the fact that only a single specimen is available for investigation. In addition, data on the biology of monoplacophorans are scarce (Menzies *et al.* 1959; Lowenstam 1978). Because the mantle cavity of *Laevipilina antarctica* is ventilated by the densely ciliated ctenidia, the epidermal bacteria are certainly in a good position to utilize dissolved organic matter (DOM) from the external environment. One might also suggest a role for the bacteria in recycling the DOM released by the host, as is the case in the bivalve *Venus verrucosa* (see review in Prieur *et al.* 1990) and as speculated also in echinoderms (Holland & Nealson 1978). The regular epidermal cells, in turn, could take up the DOM released by the bacteria or could take up whole bacteria by phagocytosis as shown in figure 10. However, the contribution of this mode of nutrient uptake and that of phagocytosis to the energy budget of *Laevipilina antarctica* cannot be estimated at present. No data are available with regard to how colonization of the offsprings by the symbionts might take place.

We thank the crew of R/V *Polarstern* for the assistance during collection. We are grateful to Reinhard and Gunde Rieger (University of Innsbruck) for very valuable informations on bacterial symbiosis among metazoans. The study was financially supported by grant P9076-BIO of the Austrian Science Foundation to G.H.

REFERENCES

Daddow, L.Y.M. 1983 A double lead stain method for enhancing contrast of ultrathin sections in electron microscopy: a modified multiple staining technique. *J. Microsc.* **129**, 147–153.

Haszprunar, G. & Schaefer, K. 1995 Monoplacophora. In *Microscopic anatomy of invertebrates*, vol. 6 (*Mollusca II*) (ed. F. W. Harrison & A. J. Kohn), New York: John Wiley & Sons. (In the press.)

Holland, N.D. & Nealson, K.H. 1978 The fine structure of the echinoderm cuticle and the subcuticular bacteria of echinoderms. *Acta Zool.* (*Stockh.*) **59**, 169–185.

Lemche, H. & Wingstrand, K.G. 1959 The anatomy of *Neopilina galatheae* Lemche, 1957. *Galathea Report* **3**, 9–71, 56 pls.

Lowenstam, H.A. 1978 Recovery, behaviour, and evolutionary implications of live Monoplacophora. *Nature, Lond.* **213**, 231–232.

McKenzie, J.D. & Kelly, M.S. 1994 Comparative study of sub-cuticular bacteria in brittlestars (Echinodermata: Ophiuroidea). *Mar. Biol.* **120**, 65–80.

Menon, J.G. & Arp, A.J. 1993 The integument of the marine echiuran worm *Urechis caupo*. *Biol. Bull.* **185**, 440–454.

Menzies, R.J., Ewing, M., Worzel, J.L. & Clarke, A.H. Jr 1959 Ecology of recent Monoplacophora. *Oikos* **10**, 168–182.

Ott, J., Rieger, G., Rieger, R.M. & Enderes, F. 1982 New mouthless interstitial worms from the sulfide system: Symbiosis with prokaryotes. *Mar. Ecol.* (*PSZNI*) **3**, 313–333.

Prieur, D., Mével, D.P., Nicolas, J.-L., Plusquellec, A. & Vigneulle, M. 1990 Interactions between bivalve molluscs and bacteria in the marine environment. *Oceanogr. Mar. Biol. A. Rev.* **28**, 277–352.

Rieger, R.M. 1984 Evolution of the cuticle in the lower Metazoa. In *Biology of the integument*, vol. 1 (*Invertebrates*) (ed. J. Bereither-Hahn, A. G. Matoltsy & K. S. Richards), pp. 389–399. Berlin: Springer Verlag.

Rieger, R.M. & Rieger, G.E. 1991 Bacterial symbionts of *Jennaria pulchra*, a new genus of interstitial worm with uncertain systematic position. *Zool.* **31**(5), 25A (#149).

Saffo, M.B. 1992 Invertebrates in endosymbiotic associations. *Am. Zool.* **32**, 557–565.

Scheltema, A., Tscherkassky, M. & Kuzirian, A.M. 1994 Aplacophora. In *Microscopic anatomy of invertebrates*, vol. 5 (*Mollusca I*) (ed. F. W. Harrison & A. J. Kohn), pp. 13–54. New York: John Wiley & Sons.

Spurr, A.R. 1969 A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**, 31–43.

Warén, A. & Hain, S. 1992 *Laevipilina antarctica* and *Micropilina arntzi*, two new monoplacophorans from the Antarctic. *Veliger* **35**, 165–176.

Wingstrand, K.G. 1985 On the anatomy and relationships of recent Monoplacophora. *Galathea Rep.* **16**, 7–94, 12 pls.

Received 23 May 1994; accepted 20 June 1994

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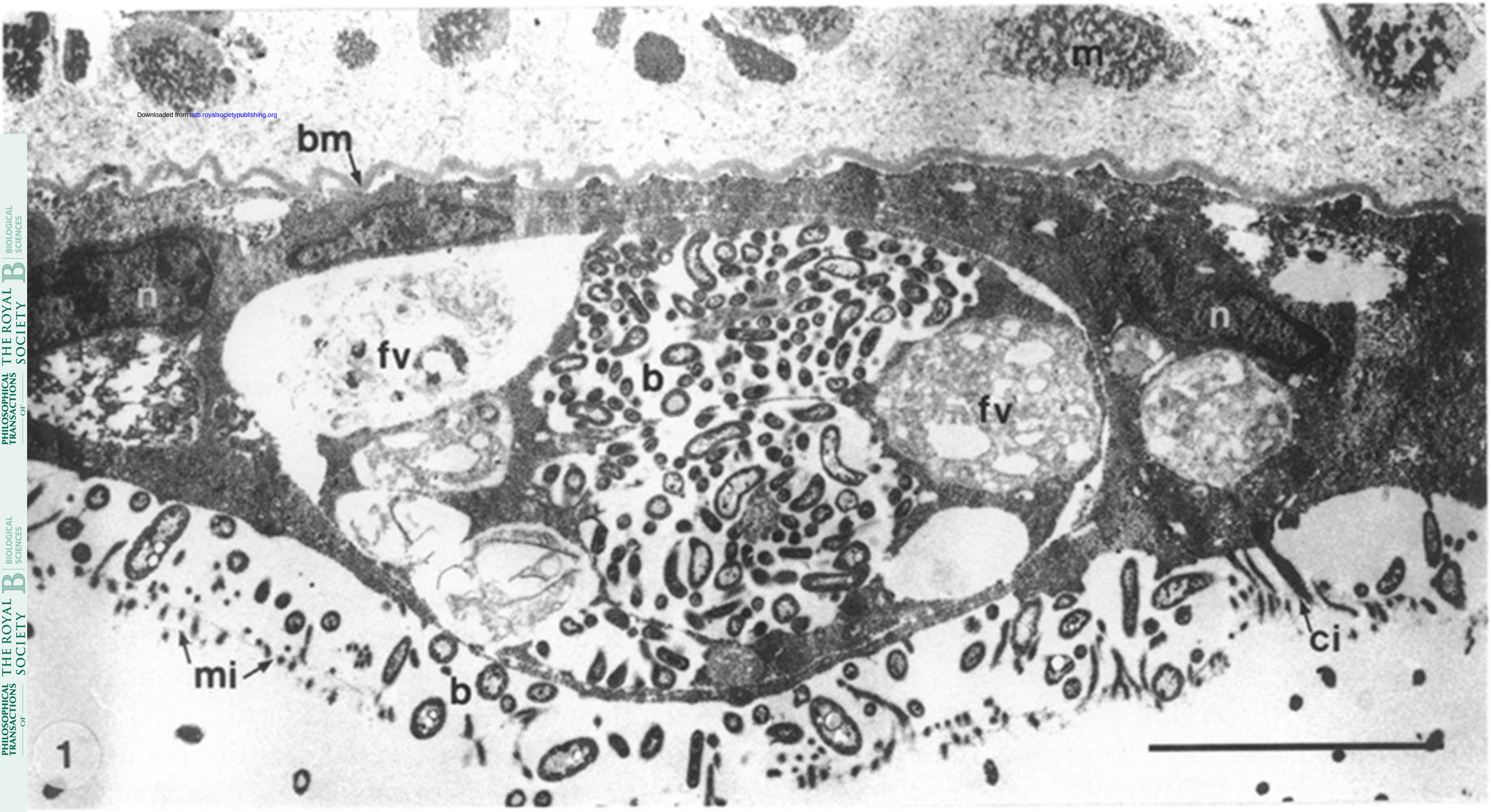

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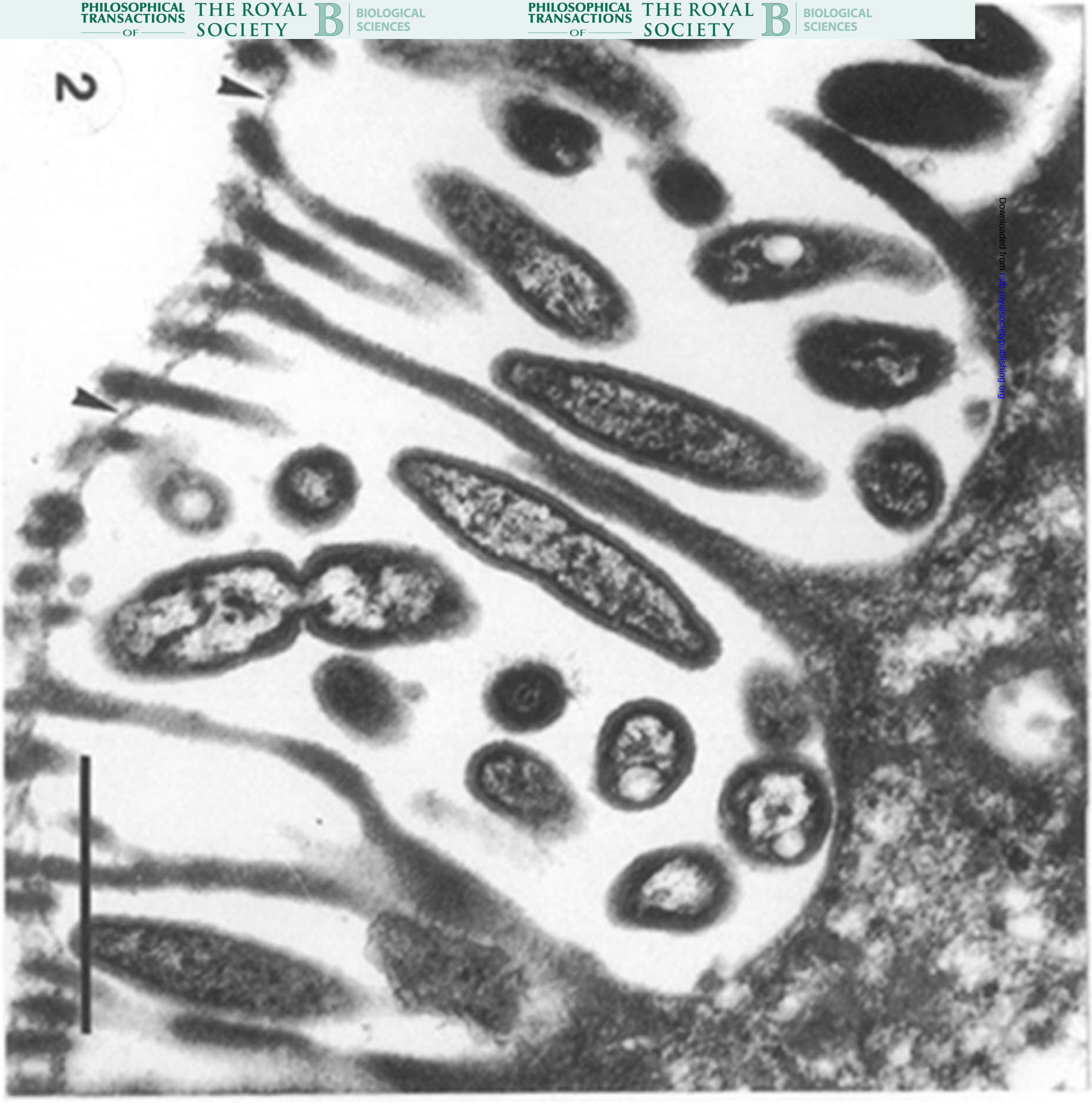

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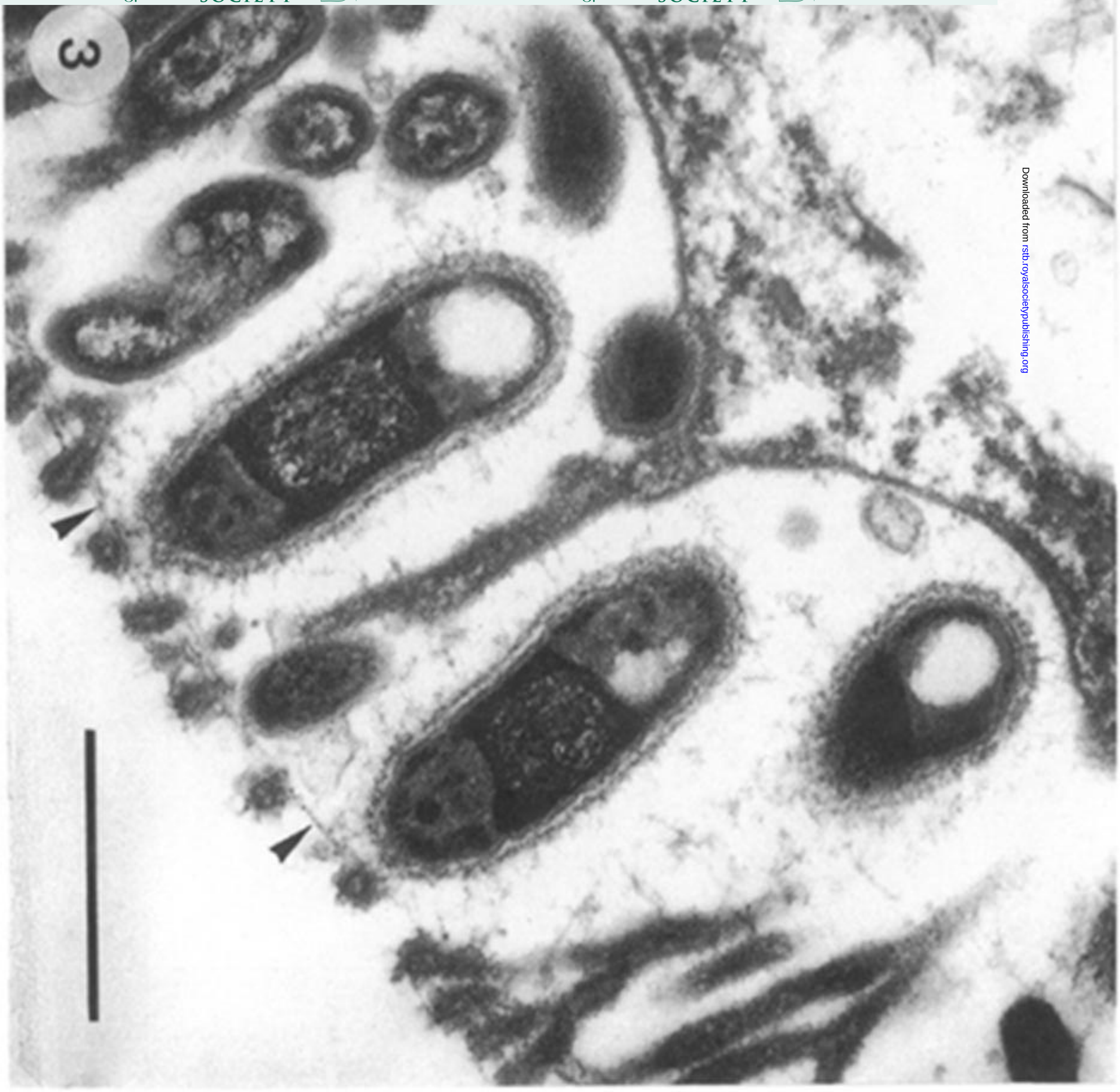

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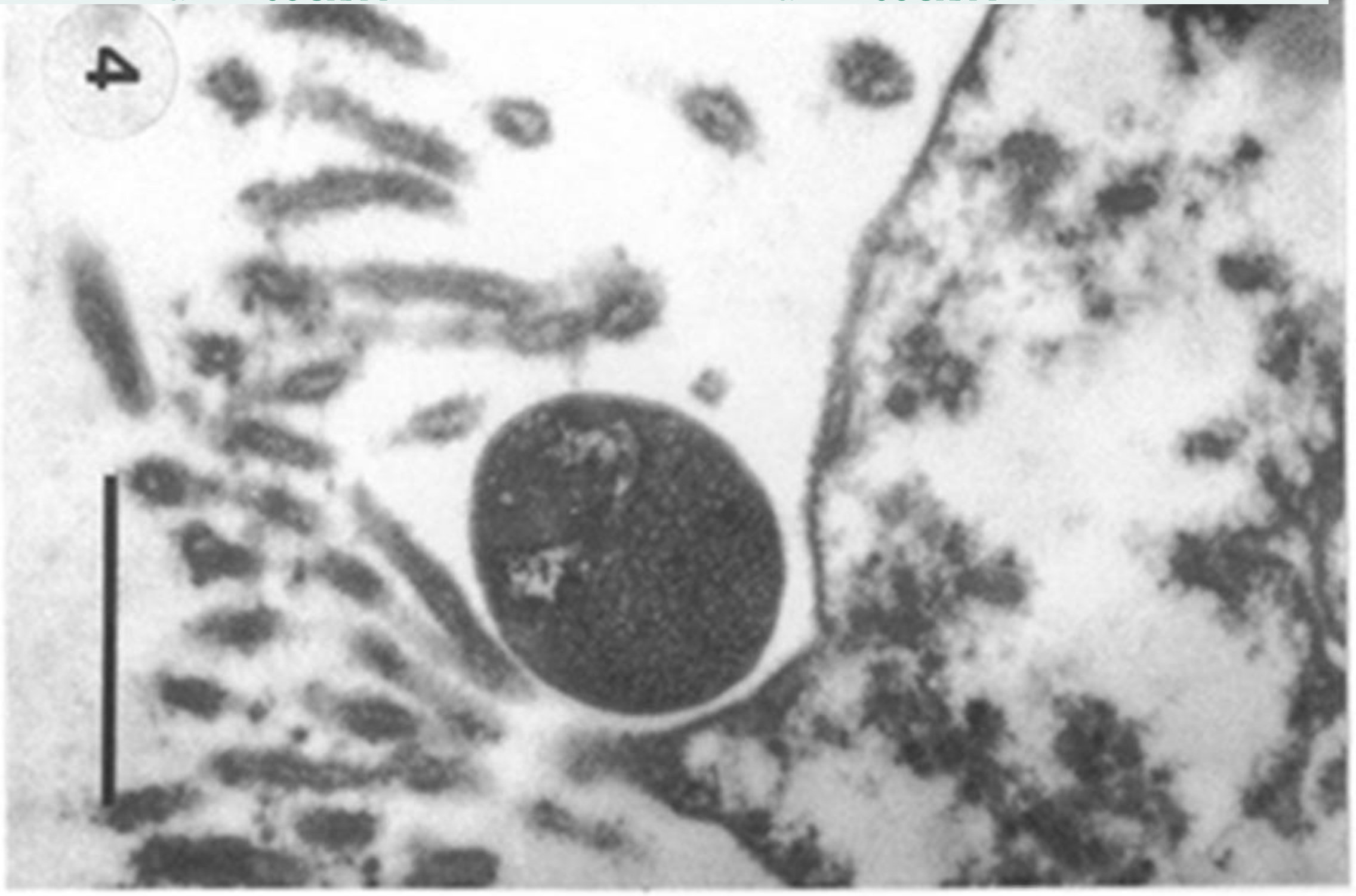

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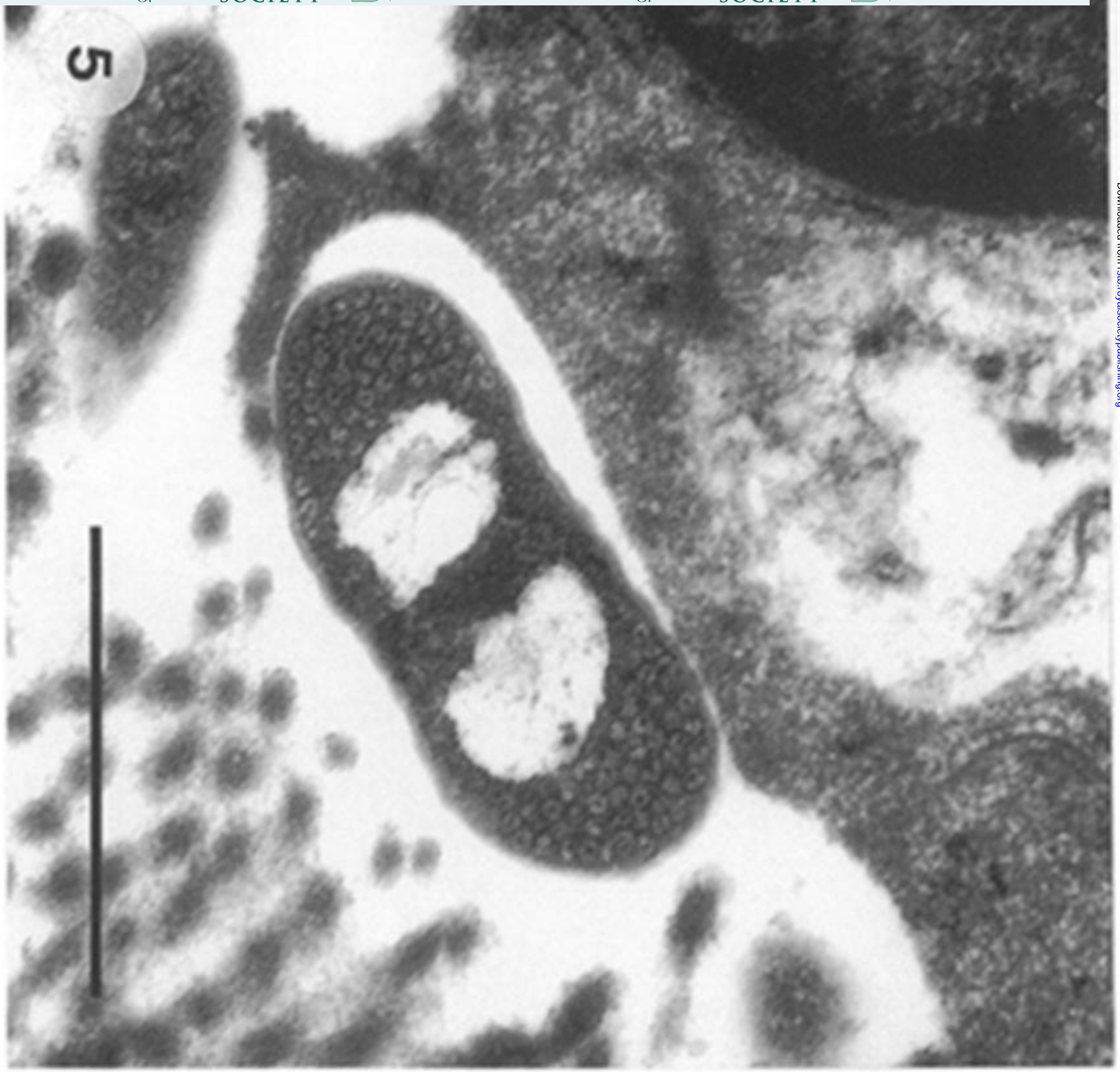

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