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Abstract

Many insect endosymbionts described so far are gram-negative bacteria. Primary endosymbionts are obligatory bacteria usually harboured by insects inside vacuoles in specialized cells called bacteriocytes. This combination produces a typical three-membrane system with one membrane derived from the insect vacuole and the other two from the bacterial gram-negative cell envelope, composed by the cell wall (the outer membrane plus the periplasmic space) and the plasma membrane (the inner membrane). For the last 21 years, the primary endosymbiont of whiteflies '*Candidatus* Portiera aleyrodidarum' was considered an exception to this rule. Previous works stated that only two membranes were present, the vacuolar membrane and one of the two bacterial membranes. The absence of the cell wall was related to the special vertical transmission of the endosymbionts in whiteflies. In this work, we present electron microscopic studies showing a complete cell envelope in '*Ca.* Portiera aleyrodidarum' from the whitefly *Bemisia tabaci*. Additionally, comparison of the inferred metabolism from the gene content did not show any difference in cell envelope biogenesis compared with the closely related three-membrane endosymbionts '*Candidatus* Carsonella ruddii' and '*Candidatus* Evansia muelleri' Xc1. Our results rule out the proposal that '*Ca.* Portiera aleyrodidarum' is an exception to the three-membrane system.

Introduction

Endosymbiotic bacteria are commonly found in insects and are required for host survival, normally providing nutrients the insect cannot synthesize or obtain from their diet. Usually, these endosymbionts have undergone a drastic genome reduction, retaining only those genes indispensable for cell functioning and maintenance and those required to fulfil their symbiotic role. Most primary endosymbionts are enclosed inside vacuoles in specialized host cells called bacteriocytes, which can form an organ-like tissue called a bacteriome, and present strict vertical transmission from mother to offspring (Baumann, 2005). Insects from the Sternorrhyncha suborder are phytopha-gous, feeding on an unbalance diet of phloem-sap, rich in carbohydrates, but very poor in nitrogenous compounds. The main function of primary endosymbionts is to complement the diet of their hosts. Most of these endosymbionts belong to gamma-proteobacteria and present a three-membrane system: one vacuolar membrane, derived from the host, and the two typical gram-negative bacterial membranes (outer and inner, separated by the periplasmic space) (Baumann, 2005).

Whiteflies (*Aleyrodidae*) belong to *Sternorrhyncha* and some species, like *Bemisia tabaci* or *Trialeurodes vaporariorum,* are important agricultural pests. All whiteflies harbour a primary endosymbiont called '*Candidatus* Portiera aleyrodidarum' that presents an extremely reduced genome and provides the insect with essential amino acids and carotenoids (Thao & Baumann, 2004; Santos-Garcia *et al.*, 2012; Sloan & Moran, 2012). '*Ca.* Portiera aleyrodidarum' was first described as a pleomorphic bacterium in *Bemisia tabaci* and *Trialeurodes vaporariorum* (Costa *et al.*, 1993). Subsequent studies showed that it was an uncommon primary endosymbiont due to the lack of a clear cell wall (Costa *et al.*, 1995; Szklarzewicz & Moskal, 2001; Coombs *et al.*, 2007). It has been postulated that the lack of a cell wall may be related to the special endosymbiont transmission

mechanism in whiteflies compared with other insects. In whiteflies, the whole bacteriocyte migrates to the oocyte through the pedicel where it changes its shape via the eukaryotic cytoskeleton. Also, 'Ca. Portiera aleyrodidarum' becomes more elongated in shape and, for this reason, a more flexible cell envelope could be needed (Szklarzewicz & Moskal, 2001; Coombs et al., 2007). By contrast, other primary endosymbionts such as Buchnera aphidicola BAp strain A5, from the aphid Acyrthosiphon pisum, which maintains a clear three-membrane system, seem to colonize the offspring bacteriocyte by an exo/ endocytosis process (Koga et al., 2012). In fact, there is only one other described case of a two-system membrane: B. aphidicola BBp from the aphid Baizongia pistaciae (Charles et al., 2011). It is also remarkable that the closest relatives to 'Ca. Portiera aleyrodidarum', 'Candidatus Carsonella ruddii' and 'Candidatus Evansia muelleri', primary endosymbiont of psyllids and moss bugs respectively, retain the three-membrane organization (Baumann, 2005; Santos-Garcia et al., 2014a).

In this work, we revisited the ultrastructure of 'Ca. Portiera aleyrodidarum' from Bemisia tabaci. Our aim is to confirm whether this endosymbiont possesses the two postulated membranes, and thus being an exception to the three-membrane system, or it is a mistake that deserves be corrected.

Materials and methods

The B. tabaci strain QHC-VLC has been maintained under laboratory conditions for 10 years. This strain harbours the primary endosymbiont 'Ca. Portiera aleyrodidarum' BT-QVLC and the secondary endosymbionts 'Candidatus Hamiltonella defensa' and 'Candidatus Cardinium hertigii' cBtQ1 (Santos- Garcia et al., 2012, 2014b). Two samples of B. tabaci QHC-VLC nymphs were collected on different days from cotton leaves with a water-floss device and briefly cleaned with 70% ethanol and dis-

tilled water. Samples were fixed in Karnowsky's fixative (2% paraformaldehyde and a 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 7.2 pH) with five steps of 1 min in a vacuum pump and left for overnight fixation at 4 °C. Samples were then washed and postfixed in 2% OsO_4 for 2 h. Then, they were washed, dehydrated through ethanol series (30, 50, 70, 90 and 100), passed to propylene oxide and embedded in LR White resin. Resin blocks were cut in a Leica Ultracut EM UC6 (60–90-nm sections), and grids were contrasted with 2% uranyl acetate and Reynolds' lead citrate. Samples were analysed under a JEOL JEM-1010 transmission electron microscope at 80 kV. Three different images from each sample clearly showing the Portiera's cell wall were used to measure the membrane components. Five measurements were taken for each membrane component from each picture with Fiji (Schindelin et al., 2012). For plotting reasons, images were cropped with InkScape. Original images will be supplied upon request.

Metabolic capabilities of 'Ca. Portiera aleyrodidarum' TV (from T. vaporariorum), 'Ca. Carsonella ruddii' HC (from Homalodisca coagulata), B. aphidicola BCc (from Cinara cedri), B. aphidicola BAp 5A (from A. pisum) and B. aphidicola BBp (from B. pistaciae) were explored in MetaCyc (Caspi et al., 2014). Additionally, metabolic capabilities of 'Ca. Portiera aleyrodidarum' BT-QVLC and 'Ca. Evansia muelleri' were obtained from previous works (Santos-Garcia et al., 2012, 2014a).

Results

Two types of 'Ca. Portiera aleyrodidarum' membrane structures were found. The most common ultrastructure obtained did not present either a distinctive cell wall or the outer membrane and was always separated from the host's vacuolar membrane (Costa et al., 1993, 1995; Szklarzewicz & Moskal, 2001; Baumann, 2005; Coombs et al., 2007) (Fig. 1). However, a less common membrane
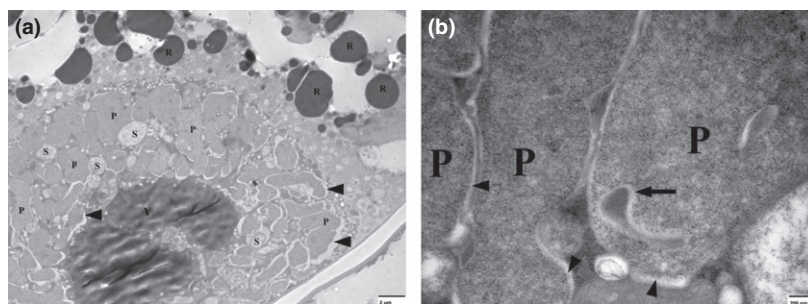

Fig. 1. Previously reported 'Ca. Portiera aleyrodidarum' ultrastructure. (a) Bacteriocytes from a Bemisia tabaci egg. Bacteriocyte is surrounded by a cell with reserve substances (R). Vitellogenic reserve (Y) is surrounded by the bacteriocytes. Primary (P) and secondary endosymbionts (S) can be seen. (b) Magnification from nymph bacteriocyte showing different 'Ca. Portiera aleyrodidarum' (P) cells without a clear cell wall. Arrowheads denote vacuolar spaces as results of 'Ca. Portiera aleyrodidarum' degradation. Arrows denote 'Ca. Portiera aleyrodidarum' membrane infoldings.

structure showing a cell wall was detected (Fig. 2a and c). This structure was composed of the host's vacuole membrane and a cell wall-like structure. Cells with the less common structure can be found attached to the vacuolar membrane or separated from it, but still with parts of the cell envelope in contact with the vacuolar membrane. In some images, the cell envelope of '*Ca.* Portiera aleyrodidarum' presented the typical structure of a gram-negative bacterium: outer membrane, periplasmic space and inner membrane (Fig. 2b and d). Even though the periplasmic space and both membranes were not always completely separated, the cell envelope showed a variable width depending on whether the periplasmic region was detectable or not (Table 1). The average width for the '*Ca.* Portiera aleyrodidarum' outer and inner membranes were 9.52 and 6.88 nm, respectively. Finally, the host's vacuolar membrane was a little bit wider than '*Ca.* Portiera aleyrodidarum' membranes (Table 1).

The biosynthetic capabilities, derived from the genome sequence, related to cell envelope biosynthesis are summarized in Table 2. Both '*Ca.* Portiera aleyrodidarum' and '*Ca.* Carsonella ruddii' (but no '*Ca.* Evansia muelleri') are able to produce cardiolipin, a phospholipid that is one of the main components of bacterial membranes. None of the three endosymbionts are able to synthesize peptidoglycan, which is a component of the cell wall (Table 2). Also two *B. aphidicola* strains (BAp 5A and BBp) are able to synthesize the whole gram-negative cell envelope. Although *B. aphidicola* BCc presents a three-membrane system, it is not able to synthesize peptidoglycan and can only produce a small range of fatty acids (Table 2).

Discussion

Almost all gram-negative primary endosymbionts reported so far are harboured in the host's vacuoles and present three membranes: the vacuolar membrane derived from the host and the two membranes that compose the gram-negative cell envelope, the outer and the inner
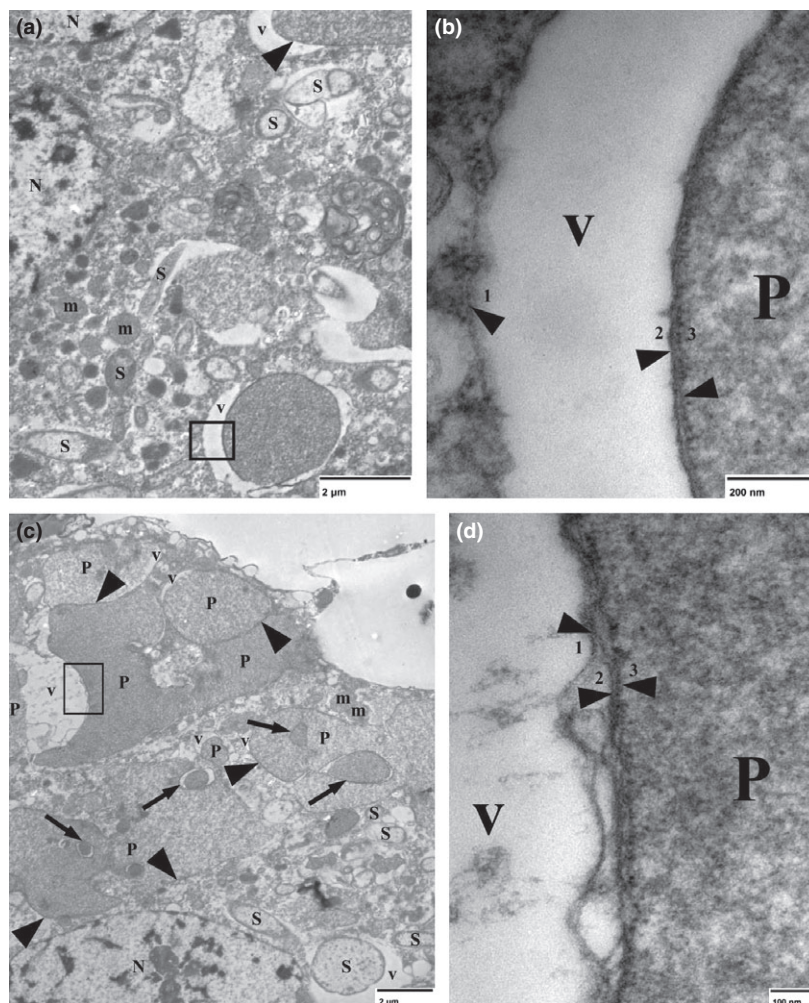

Fig. 2. '*Ca.* Portiera aleyrodidarum' three-membrane structure. Bacteriocytes from *Bemisia tabaci* nymphs. (a and c) general view of nymphal bacteriocytes. Some secondary endosymbionts (S), mitochondria (m) and nuclei (N) are observed. Vacuolar spaces (V) can be seen, but '*Ca.* Portiera aleyrodidarum' (P) cells still conserve a clear cell envelope (Arrowheads). Black boxes denote magnified area. (b and d) magnified areas showing the three-membrane system of '*Ca.* Portiera aleyrodidarum'. Arrowheads points to the different membranes: (1) vacuolar membrane, (2) outer membrane, (3) inner membrane.

Table 1. '*Ca.* Portiera aleyrodidarum' membrane measurements

	Mean (nm)	Geometric mean (nm)	Standard deviation (nm)
Outer membrane	9.52	9.26	2.20
Inner membrane	7.72	7.39	2.34
Periplasmic space	6.88	6.36	2.59
Outer + Inner + Peripl. space	30.18	29.52	6.69
Outer + Inner	21.12	20.47	5.40
Vacuole membrane	12.00	11.67	2.81

Table 2. Simplified membrane biosynthesis capabilities

Species	Peptidoglycan	Cardiolipin	Other fatty acids/lipids
B. aphidicola BCc	−	−	+
B. aphidicola BAp5A	+	+	+
B. aphidicola BBp	+	+	−
'*Ca.* Carsonella ruddii' HC	−	+	−
'*Ca.* Evansia muelleri' Xc1	−	−	−
'*Ca.* Portiera aleyrodidarum' BT-VLC	−	+	−
'*Ca.* Portiera aleyrodidarum' TV	−	+	−

+: presence; −: absence.

membrane. For many years, '*Ca.* Portiera aleyrodidarum' was proposed to be an exception for the three-membrane system in endosymbionts of insects. No differences were encountered when the putative metabolic capabilities of this endosymbiont were compared to those of other primary endosymbionts with three membranes. It is true that larger (in genome size terms) primary endosymbionts, like *B. aphidicola* BAp 5A, retain the ability to synthesize a minimal cell envelope with all its parts clearly distinguishable, but also the reduced *B. aphidicola* BCc genome possesses the three clearly visible membranes (Charles *et al.*, 2011). It has been postulated that this *Buchnera* might be using the metabolites from the co-obligate endosymbiont '*Candidatus* Serratia symbiotica' to produce its cell envelope (Lamelas *et al.*, 2011). At present, the only other reported case of a two-membrane system was that of *B. aphidicola* BBp (Charles *et al.*, 2011). However, taking into account that according to its putative metabolic capabilities it is able to synthesize the two gram-negative membranes, and considering our results with '*Ca.* Portiera aleyrodidarum' in this work, we cannot rule out that this system may be an artefact. Thus, similar to '*Ca.* Portiera aleyrodidarum', its ultrastructure should be revisited to confirm its membrane organization. On the other hand, '*Ca.* Carsonella ruddii', derived from the same ancestral symbiotic infection event than '*Ca.* Portiera aleyrodidarum', has an even more reduced genome but maintains the three-membrane structure and has the same cell wall biogenesis capabilities as the latter (Bau-

mann, 2005; Santos-Garcia *et al.*, 2014a). In addition, '*Ca.* Evansia muelleri', also conserves the three-membrane structure even lacking the ability to produce cardiolipin (Santos-Garcia *et al.*, 2014a). Lastly, '*Ca.* Portiera aleyrodidarum', '*Ca.* Carsonella ruddii' and '*Ca.* Evansia muelleri' cannot synthesize peptidoglycan, and thus, we would expect a reduced or absent periplasmic space. Because peptidoglycan is responsible for supplying mechanical force (and resistance to different environmental stresses), the cell envelopes of these endosymbionts must be extremely fragile, and their integrity probably depends on the maintenance of an intact host's vacuole. Although it is still unclear how extremely reduced primary endosymbionts lacking most of the cell envelope biosynthetic genes produce their membranes, there are suggestions that it could be through the host's control mechanism or the use of host-derived membranes (Husnik *et al.*, 2013). Additionally, as stated before for *B. aphidicola* BCc, a second endosymbiont could provide the lacking cell envelope biogenesis functions in '*Ca.* Portiera aleyrodidarum' ('*Ca.* Hamiltonella defensa' in the case of '*Ca.* Portiera aleyrodidarum' BT-QVLC),

When transmission electron pictures from '*Ca.* Carsonella ruddii', '*Ca.* Evansia muelleri' and '*Ca.* Portiera aleyrodidarum' are compared, only the latter show big vacuolar spaces, as result of the separation of the endosymbiont from the vacuolar membrane (Waku & Endo, 1987; Costa *et al.*, 1993, 1995; Thao *et al.*, 2000; Szklarzewicz & Moskal, 2001; Coombs *et al.*, 2007; Kuechler *et al.*, 2013) (Fig. 1). Although this could be an indication of a more degraded stage of the bacteriocyte and a fragile cell envelope in '*Ca.* Portiera aleyrodidarum', our proposal is that the absence of the three-membrane system is a technical artefact, produced by the difficulty in obtaining good fixed samples. In fact, in well-conserved samples, with '*Ca.* Portiera aleyrodidarum' still in contact with the vacuolar membrane, the cell envelope is observed (Fig. 2). However, the components of the cell envelope could only be observed in some of the specimens with membranes in an initial state of degradation (Fig. 2b and d). Also, our results suggest that no peptidoglycan (or only very small amounts from and unknown source) is deposited in the periplasmic space because it is not clearly defined in nondegraded cell envelopes. This result is also confirmed by the metabolic capabilities of '*Ca.* Portiera aleyrodidarum'. We cannot confirm or refute that it uses compounds from the secondary symbionts that share the bacteriocytes or that a complementation/regulation may exist with the host (Santos-Garcia *et al.*, 2012, 2014b; Husnik *et al.*, 2013).

In conclusion, by combining ultrastructural and metabolic analyses, we have demonstrated that '*Ca.* Portiera aleyrodidarum' possesses the canonical three-membrane

system, although its cell envelope is more fragile than that of the closely related endosymbionts 'Ca. Carsonella ruddii' and 'Ca. Evansia muelleri'. This fragility could be related to the transmission route of endosymbionts in whiteflies as previously proposed (Szklarzewicz & Moskal, 2001; Coombs *et al.*, 2007). Without experimental procedures to determine endosymbionts' membrane composition, we can only speculate that these differences could be due to small changes in phospholipid composition in the membranes, probably obtained from the host's cytosol.

Acknowledgements

References

Baumann P (2005) Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu Rev Microbiol* **59**: 155–189.

Caspi R, Altman T, Billington R *et al.* (2014) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. *Nucleic Acids Res* **42**: D459–D471.

Charles H, Balmand S, Lamelas A *et al.* (2011) A genomic reappraisal of symbiotic function in the Aphid/*Buchnera* symbiosis: reduced transporter sets and variable membrane organisations. *PLoS ONE* **6**: e29096.

Coombs MT, Costa HS, De Barro P & Rosell RC (2007) Pre-imaginal egg maturation and bacteriocyte inclusion in *Bemisia* aff. *gigantea* (Hemiptera: Aleyrodidae). *Ann Entomol Soc Am* **100**: 736–744.

Costa HS, Westcot DM, Ullman DE & Johnson MW (1993) Ultrastructure of the endosymbionts of the whitefly, *Bemisia tabaci* and *Trialeurodes vaporariorum*. *Protoplasma* **176**: 106–115.

Costa HS, Westcot DM, Ullman DE, Rosell R, Brown JK & Johnson MW (1995) Morphological variation in *Bemisia* endosymbionts. *Protoplasma* **189**: 194–202.

Husnik F, Nikoh N, Koga R *et al.* (2013) Horizontal gene transfer from diverse bacteria to an insect genome enables a tripartite nested mealybug symbiosis. *Cell* **153**: 1567–1578.

Koga R, Meng XY, Tsuchida T & Fukatsu T (2012) Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. *P Natl Acad Sci USA* **109**: 1230–1237.

Kuechler SM, Gibbs G, Burckhardt D, Dettner K & Hartung V (2013) Diversity of bacterial endosymbionts and bacteria-host co-evolution in Gondwanan relict moss bugs (*Hemiptera*: *Coleorrhyncha*: *Peloridiidae*). *Environ Microbiol* **15**: 2031–2042.

Lamelas A, Gosalbes MJ, Manzano-Marìn A, Peretó J, Moya A & Latorre A (2011) *Serratia symbiotica* from the aphid *Cinara cedri*: a missing link from facultative to obligate insect endosymbiont. *PLoS Genet* **7**: e1002357.

Santos-Garcia D, Farnier PA, Beitia F, Zchori-Fein E, Vavre F, Mouton L, Moya A, Latorre A & Silva FJ (2012) Complete genome sequence of "*Candidatus* Portiera aleyrodidarum" BT-QVLC, an obligate symbiont that supplies amino acids and carotenoids to *Bemisia tabaci*. *J Bacteriol* **194**: 6654–6655.

Santos-Garcia D, Latorre A, Moya A, Gibbs G, Hartung V, Dettner K, Kuechler SM & Silva FJ (2014a) Small but powerful, the primary endosymbiont of moss bugs, *Candidatus* Evansia muelleri, holds a reduced genome with large biosynthetic capabilities. *Genome Biol Evol* **6**: 1875–1893.

Santos-Garcia D, Rollat-Farnier PA, Beitia F, Zchori-Fein E, Vavre F, Mouton L, Moya A, Latorre A & Silva FJ (2014b) The genome of *Cardinium* cBtQ1 provides insights into genome reduction, symbiont motility and its settlement in *Bemisia tabaci*. *Genome Biol Evol* **6**: 1013–1030.

Schindelin J, Arganda-Carreras I, Frise E *et al.* (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**: 676–682.

Sloan DB & Moran NA (2012) Endosymbiotic bacteria as a source of carotenoids in whiteflies. *Biol Lett* **8**: 986–989.

Szklarzewicz T & Moskal A (2001) Ultrastructure, distribution, and transmission of endosymbionts in the whitefly *Aleurochiton aceris* Modeer (*Insecta*, *Hemiptera*, *Aleyrodinea*). *Protoplasma* **218**: 45–53.

Thao MLL & Baumann P (2004) Evolutionary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts. *Appl Environ Microbiol* **70**: 3401–3406.

Thao ML, Moran NA, Abbot P, Brennan EB, Burckhardt DH & Baumann P (2000) Cospeciation of psyllids and their primary prokaryotic endosymbionts. *Appl Environ Microbiol* **66**: 2898–2905.

Waku Y & Endo Y (1987) Ultrastructure and life cycle of the symbionts in a homopteran insect, *Anomoneura mori* Schwartz (*Psyllidae*). *Appl Entomol Zool (Jpn)* **22**: 630–637.