Symbiosis, 14 (1992) 475–484
Balaban, Philadelphia/Rehovot

Ultrastructural Analysis of an Artificial Alga-Bacterium Endosymbiosis After Prolonged Cultivation

I. GYURJÁN*, P. KORÀNYI* and GY. PALESS**
[1]*Departments of Plant Anatomy and* [2]*Genetics, Eötvös Lóránd University*
Budapest, Pushkin u. 11, H-1088, Hungary
Tel. 118-0021

Received September 5, 1991; Accepted February 13, 1992

Abstract

Artificial endosymbiotic partnerships were established between different diazotrophic *Azotobacter* species and unicellular green alga *Chlamydomonas reinhardtii* mutants. During the five-year cultivation on nitrogen- and carbohydrate-free medium one (*C. reinhardtii* 380×354 + *A. vinelandii* CCM 289) of the mixed systems showed exceptional stability and relatively fast and balanced autotrophic growth. Electron microscopy revealed that at least 10% of the algae harbored symbionts and the alga/bacterium volume ratio is constant. In micrographs, bacteria are seen in membrane-bounded vesicles inside the host cytoplasm, between the cell wall and plasma membrane, in or across the cell wall, and in the intercellular spaces. The relative numbers of symbionts in the different host compartments are not random. The regulation of their quantity may be a prerequisite of the balanced growth of this endosymbiotic system.

Keywords: *Azotobacter*, *Chlamydomonas*, intracellular symbiosis, electron microscopy, symbiont number and regulation

1. Introduction

The idea of an artificial cellular symbiosis was based on the observations that many organisms live together with natural symbionts and several cell types or organelles are the result of endocytobiotic events. In the early 1980s

forced prokaryote-eukaryote associations and endosymbioses were established and these systems were capable both of photosynthesis and nitrogen fixation (Gyurján et al., 1984b; Nghia et al., 1986a,b,c,). It was shown previously that mixed cultures of *Chlamydomonas* and *Azotobacter* are able to live continuously on nitrogen- and carbohydrate-free medium in the form of an intercellular association (Gyurján et al., 1984a, 1986). The eukaryotic host can meet the energy requirements of nitrogen fixation by supplying carbohydrates to the diazotrophic symbiont which in turn makes available a portion of its fixed ammonia for the host (Gyurján et al., 1984b; Nghia et al., 1986b). During the years of cultivation an increasing stability was observed in these associations which may be a consequence of mutual metabolic dependence (Korányi et al., 1990). In this case stability was characterized by the integrity of photosynthetic pigments, the metabolic interactions, and the balanced growth of the partners which means that they do not overgrow each other.

Based on the success with exocytobioses, trials were made to introduce azotobacters directly into auxotrophic cell-wall mutant algal hosts during thier polyethylene glycol (PEG)-induced fusion (Nghia et al., 1986a, 1987). Six successful partnerships were established between different arginine-requiring algal cell-wall mutants and *Azotobacter* species which are the oldest known artificial nitrogen-fixing endocytobioses kept continuously in nitrogen- and carbohydrate-free medium. We report here the electron microscopic analysis of the best five-year old system.

2. Materials and Methods

Algal culture

Arginine-requiring cell wall-less strains 308 (CW_d arg-7-3, mt^+) and 354 (CW15 arg-7 sm_1, mt^+) of unicellular green alga *Chlamydomonas reinhardtii* (Dang) were provided by R.F. Matagne (Laboratory of Molecular Genetics, University of Liege). The cells were grown at 25°C on TAP medium containing KNO_3 as a nitrogen source (Sager and Granick, 1953).

Bacterial culture

The CCM 289 stain (Purkinje University, Brno) of aerobic *Azotobacter vinelandii* (Lipman) was selected as a nitrogen fixer and used in the experiments. The cells were grown in nitrogen-free liquid and solid cultures (Newton et al., 1953).

Establishment of alga-bacterium endocytobiosis

The two complementing arginine auxotrophic cell-wall mutants of *Chlamydomonas reinhardtii* and the bacteria were used in the somatic fusion experiments as described earlier (Nghia et al., 1986a, 1987). Symbiotic cultures were maintained on a nitrogen- and carbohydrate-free medium (Gyurján et al., 1984b). They were transferred to fresh medium at least monthly.

Electron microscopy

Harvested cells were fixed, embedded, sectioned and examined together with quantitative stereology as described earlier (Korányi et al., 1990). Random sections were made regularly and several hundred micrographs were analyzed. This means the examination of about 1000 algal cells. In each stereological analysis 90 sections were randomly selected and 1800 intersections were counted on the micrographs.

Besides symbiotic algae and bacteria axenic algal cultures and reisolated bacteria were regularly examined as controls.

Detection of bacterial cellulase activity

The solid medium used for the cultivation of symbioses was supplemented with 1% carboxymethylcellulose (CMC). After 10 d incubation, cellulase activity was detected with 0.1% Congo Red solution. For quantitative determination of induced cellulase activity bacteria were cultivated in liquid medium containing 1% cellulose powder. After 10 d incubation, culture filtrates were assayed by the Mandels' filter paper method as described by Ghose et al. (1981). The value of cellulase activity (1 U = 1 μmol glucose liberated min^{-1}) characterizes the cells at 1.0 ml^{-1} optical density measured at 420 nm.

3. Results

Figures 1–4 show the ultrastructure of the five-year old alga-bacterium endocytobiosis. On the micrographs, endocytobiotic bacteria are mostly located between the cell wall and plasma membrane (Fig. 1), and generally 1 or 2 bacteria are present inside the cytoplasm in membrane-bounded vesicles, like true organelles (Fig. 2). The maximum number of intracellular bacteria in one alga was 8 in the sectioned area. About 10% of the examined algal cells harbored endocytobionts at the level of section. On average 5–10 times more azotobacters are present between the cell wall and plasma membrane than in the cytoplasm. These figures may be minimal values because serial sections were

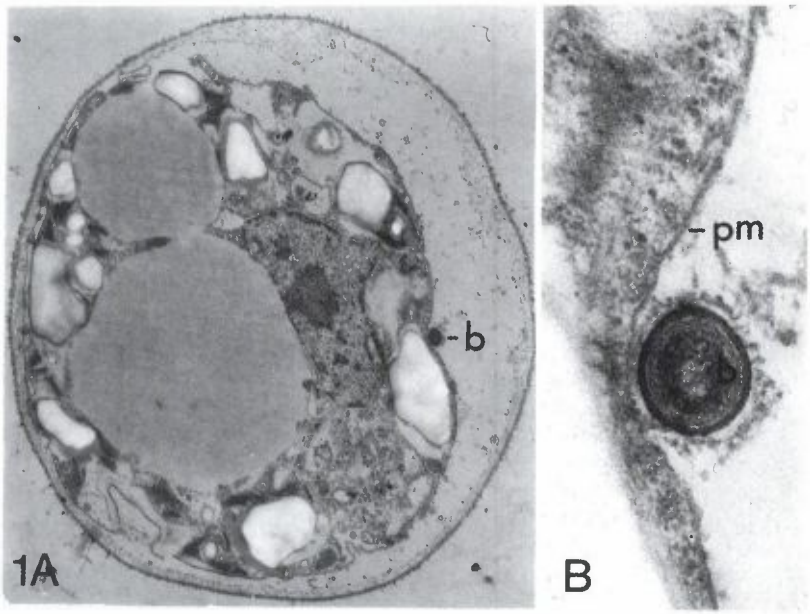

Figure 1. A. Electron micrograph of a *Chlamydomonas reinhardtii* cell harboring a bacterium between the plasma membrane and cell wall. ×7,000
B. Higher magnification of the cyst-like bacterium adhering to the plasma membrane and surrounded by a dense material which is frequently observed around symbiotic *Azotobacters*. ×145,000. (b = bacterium, pm = plasma membrane).

not made during the examinations. Some bacteria were observed attached closely to the outer surface of the plasma membrane (Fig. 1) or directly in the cell wall (Fig. 3), while dead alga cells and their surroundings are full of bacteria (Fig. 4). Previous studies showed that they are also present inside the old cell-wall after the division of algae (Nghia et al., 1986c). Based on these ultrastructural observations, they can get out during the division or destruction of their hosts and through the algal plasma membrane and cell wall.

During prolonged cultivation, bacteria are also always observed in the intercellular spaces of alga colonies albeit originally only intracytoplasmic ones were present due to the used fusion and selection techniques. Quantitative stereological analysis showed that the mean alga/intercellular bacterium volume ratios are nearly constant (47.2±2.4), however bigger than in the well characterized ectocytobiotic association (Korányi et al., 1990).

Symbiotic and reisolated bacteria showed typical azotobacter characteristics.

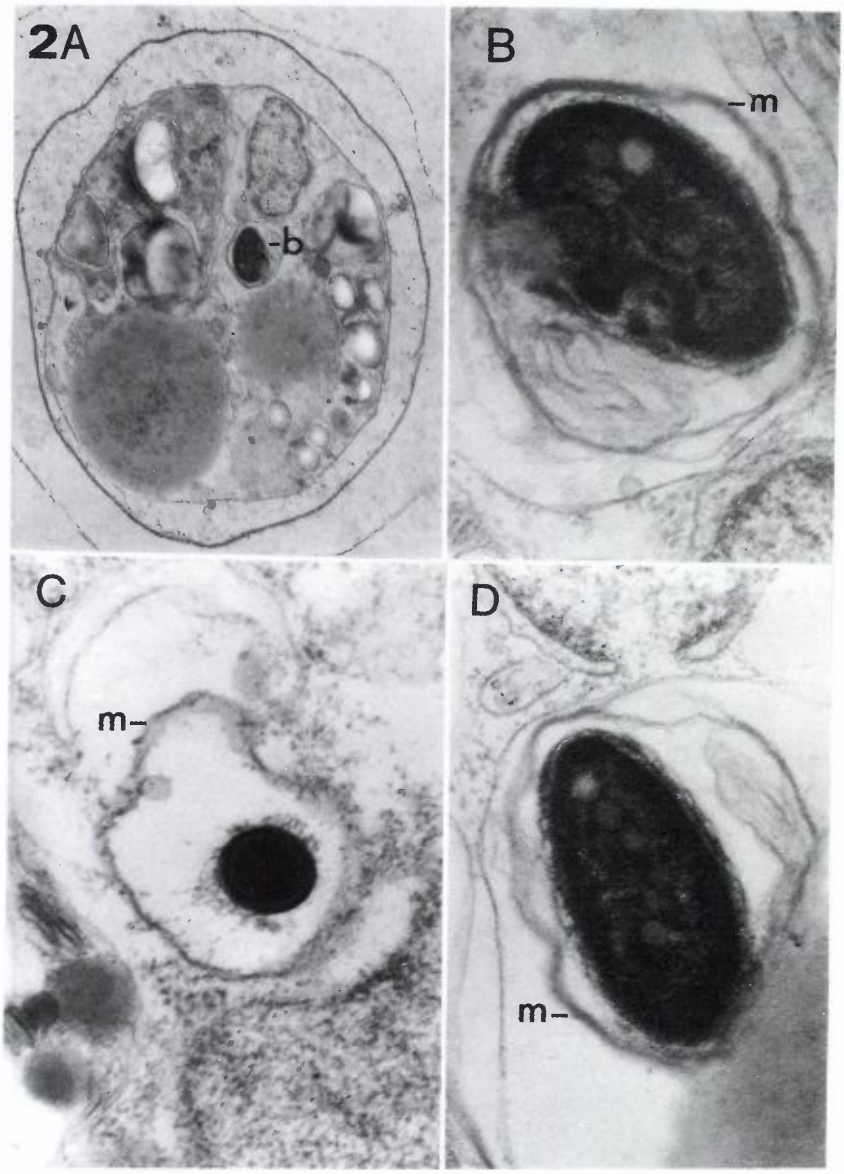

Figure 2. Elèctron micrographs of sections of endocytobiotic *Azotobacter vinelandii* cells. A. Bacterium cell in a membrane-bound vesicle inside the host cytoplasm. ×14,200. B, C, D. Appearance of intracellular bacteria in large vesicles embedded with fibrous structures of unknown nature. B, D. Longitudinal (×98,800 and ×98,500 respectively) and C transverse (×125,000) sections of intracellular bacteria. (b = bacterium, m = membrane of the vesicle).

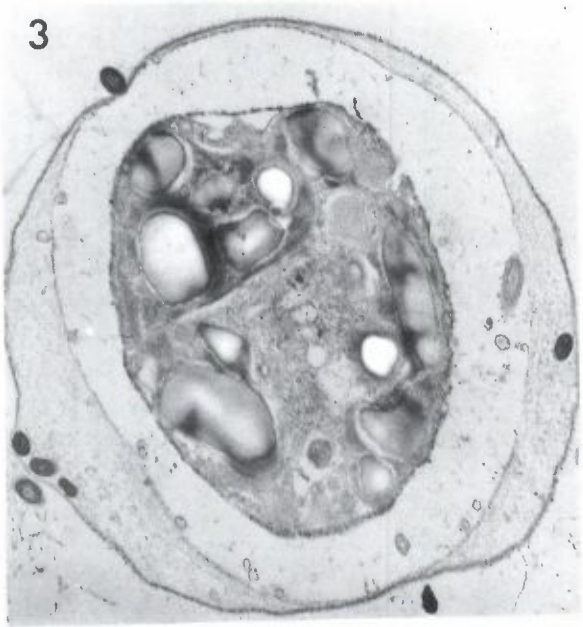

Figure 3. Bacteria inside the algal cell wall. Some bacteria are also seen outside the alga. ×11,250.

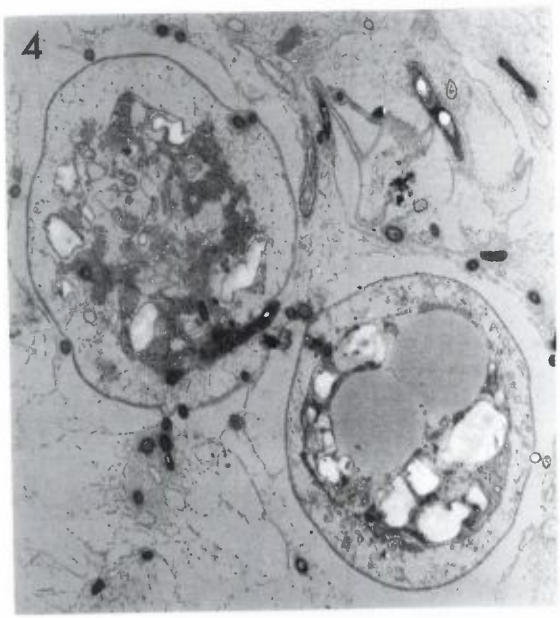

Figure 4. Electron micrograph of three disintegrating algae showing more or less damaged membrane structures. Bacteria are seen in great numbers in and around the dead host cells. ×10,800.

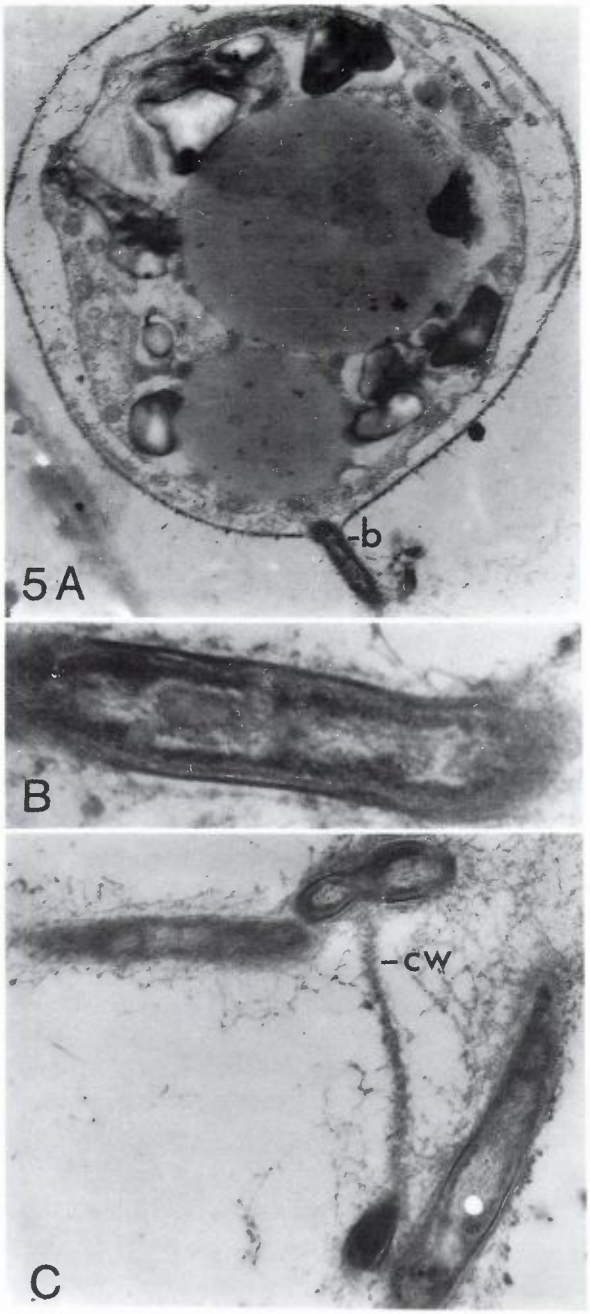

Figure 5. A. Electron micrograph of a *Chlamydomonas reinhardtii* cell with a rod-like bacterium across its cell wall. ×13,000.
B. At higher magnification, it is observed that the algal cell wall is interrupted close beside the bacterium. ×90,750.
C. Bacteria in the pores and in the neighbourhood of the cell wall. The amorphous material around them may be a product of the digestion of the algal cell wall. ×43,000. (b = bacterium, cw = cell wall of the alga).

Vegetative cells, cysts (e.g. on Fig. 1B) and germinating cysts (e.g. on Fig. 5C) were frequently observed. Reisolated bacteria grow very slowly and can be cultivated only on media supplemented with nitrogen. Bacteria have never been observed in our axenic algal cultures.

Congo Red test revealed the degradation of cellulose under the bacterium colonies. The inducible cellulase activity in liquid cultures was 0.063 ± 0.008 U.

4. Discussion

During prolonged cultivation, the alga-bacterium endocytobioses could grow on nitrogen- and carbohydrate-free medium which proves that a viable autotrophic nitrogen fixing system was established. The rate of growth at the start was low and later increased but did not reach that of the asymbiotic algae. It was not possible to make exact comparisons because asymbiotic algae and bacteria can not grow in the used selective medium.

Electron micrographs showed that many bacteria are located between the algal plasma membrane and the newly synthesized cell wall. This may be explained by an exclusion process which regulates the number of intracytoplasmic cytobionts. The multiplication of azotobacters even in this system may be more frequent than the division of algae, and their excess is removed between the cell wall and plasma membrane where the bacterial growth is also limited. Bacteria were often seen adhering to the plasma membrane and surrounded by an electron dense material (e.g. on Fig. 1). Exocytosis is a possible process by which they leave the host cytoplasm and the fibrous material present originally inside the intracytoplasmic vesicles is also expelled during this event. Jeon (1983) also suggested that an expulsion process controls the number of endocytobionts in the amoeba-bacterium association and the growth of symbionts is regulated. Exocytosis may be a mechanism in the regulation of endocytobiont number.

The five-year old endosymbiosis contains more intracellular bacteria than the selected system after the fusion process. However, the growth of partners seems to be limited and balanced. On the other hand, the relative volume ratio of intercellular bacteria is less than in a stable ectocytobiosis. It is obvious that some mechanisms regulate the relative quantities of partners during the attainment and maintenance of balanced growth and after some time an equilibrium is achieved. Bacterial overgrowth was observed only in or around dead alga cells or when glucose was added to the medium. The addition of acetate caused the overproduction of algae.

Bacteria were sometimes detected directly across the algal cell wall (Fig. 5), which may mean that the cell wall of hybrids is not continuous and bacteria can

move through the pores; or the bacteria can digest the wall. There is no experimental evidence in favor of the first explanation and pores were observed only in the neighborhood of azotobacters. The inducible production of cellulases and other hydrolases in the case of *Azotobacter* species (Samanta et al., 1989) may explain this phenomenon which was never observed in the alga-bacterium exocytobioses. This suggests that the direction of bacterial movement is outward from the host cell. Root hair invasion by *Rhizobia* and the concomitant infection thread formation involve coordinated bacterial cellulase and pectinase activities (e.g. Verma and Long, 1983). A similar enzymatic mechanism may direct the exclusion of surplus azotobacters ensuring the maintenance of a balanced symbiotic partnership. In the case of CCM 289 strain, we could detect inducible cellulase activity, however its possible *in vitro* regulation is obscure.

Our findings proved that at least 10% of the algae harbor intracytoplasmic bacteria after prolonged cultivation, and extracytoplasmic and intercellular symbionts are also always present. Thus, this endosymbiotic system is considered as a mixed endo-, exocytobiosis where each symbiont population may have its role in the nitrogen supply of the algae. It was also suggested that different (transmembrane and transwall) transfer mechanisms regulate the number of symbionts in the diazotrophic *Chlamydomonas-Azotobacter* system. Most of the bacteria are extracytoplasmic and their relative amounts are not random in the different locations. So far, it has not been possible to determine what initiates the bacterial movements and to localize diazotrophy to a specific group of symbiotic bacteria. However, the results proved that a stable and regulated organellar-type transfer of nitrogen-fixing ability is possible into eukaryotic cells.

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