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Characterisation of an Artificial Nitrogen-Fixing Alga-Bacterium Ectocytobiosis

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Abstract

Mixed cultures of nitrogen-fixing *Azotobacter vinelandii* (Lipman) and unicellular green alga *Chlamydomonas reinhardtii* (Dang) were maintained in nitrogen- and carbohydrate-free medium for five years. The ectocytobiotic colonies retained their green colour and grew autotrophically. Experimental results showed that the algae were capable of photosynthesis and the azotobacters could fix atmospheric nitrogen in the forced associations. Electron micrographs show that the bacteria are located near the surface of algal cells, in spaces between cells and sometimes inside the cytoplasm of the alga. Both the bacterial and algal cells could grow and divide in the symbiosis.

Keywords: *Azotobacter*, *Chlamydomonas*, forced association, nitrogen fixation, ectocytobiosis

1. Introduction

Recently there has been a great interest in studies of different natural symbioses at the cellular and subcellular levels. Much less effort has been made regarding the establishment and comparative analysis of artificial partnerships, although the results from such studies can provide a useful framework for the better understanding of natural systems. In this respect the spontaneous and induced amoeba-bacterium symbioses have an outstanding importance (Jeon,

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1972; 1983a,b; Jeon and Ahn, 1978). They provided evidence for the establishment of stable, integrated endocytobiosis between originally non-symbiotic partners within a relatively short period of time.

The examination of artificial nitrogen-fixing bacterium-alga associations was started in the early 1980s (Gyurján et al., 1984a,b) to study the symbiotic relations at the cellular level. Originally, the experimental system was chosen not for its possible practical but much more for its biological importance. The wide-spread occurrence of natural nitrogen-fixing symbioses gives reasons for laboratory selection or analysis of such types of systems. Several trials were made to set up artificial symbioses mainly between rhizobia and cultured plant cells. Protoplast and fusion techniques are also widely used (for a summary see Nghia and Gyurján, 1987). However, the so-called "forced associations" may be viewed as more natural systems because the partners are simply grown and maintained in the same culture, where the survivors can be easily selected. In this way a successful symbiosis was established between the nitrogen-fixing *Azotobacter vinelandii* and *Daucus carota* tissue culture (Carlson and Chaleff, 1974a,b; Chaleff and Carlson, 1975) or the diazotrophic *Azospirillum brasilense* and sugarcane callus (Berg et al., 1979, 1980; Vasil et al., 1979). One of our previous papers deals with the comparative analysis of nitrogen-fixing natural and artificial cytobioses (Korányi et al., 1988).

In our artificial cytobioses the intact partners are forced into autotrophic associations on a selective, nitrogen- and carbodydrate-free medium. In the initial test system the partners must be well-known, free-living organisms which are also widely used in laboratory experiments. In this respect diazotrophic *Azotobacter vinelandii* and photosynthetic *Chlamydomonas reinhardtii* proved to be appropriate objects. *Azotobacter* species form loose associations with certain plants and show some kind of host specificity. They are aerobic prokaryotes with a significant nitrogen-fixing capacity and can be forced into association with plant cells. The use of *Chlamydomonas reinhardtii* as the eukaryotic partner has obvious advantages. It is a unicellular fast-growing green alga with a substantial cellular surface and photosynthetic activity. The colour of the colonies is a very sensitive indicator of nitrogen supply. In case of serious nitrogen shortage their green colour turns yellow and the algae die within months.

A functioning *Azotobacter-Chlamydomonas* partnership can be used as a model of natural nitrogen-fixing prokaryote-eukaryote symbioses. The work described in this paper was carried out to establish, select, maintain and investigate autotrophic nitrogen-fixing alga-bacterium associations which are functionally stable for a long period of time.

2. Materials and Methods

Algal culture

The 137c wild-type strain (Loppes and Matagne, University of Liege) of *Chlamydomonas reinhardtii* was used. The cells were grown at 25°C on a minimal medium (Sager and Granick, 1953).

Bacterial culture

Azotobacter vinelandii CCM 289 (Purkinje University, Brno) was selected from many strains of different *Azotobacter* species as a good nitrogen-fixer (producing 1.39 nmol ethylene/10^8 cells·hr). The cells were grown in nitrogen-free medium, in liquid culture (Newton et al., 1953) at 28°C.

Establishment of bacterium-alga symbiosis

Chlamydomonas cells ($10^7 - 10^8$) were mixed with $10^9 - 10^{10}$ *Azotobacter* cells in liquid minimal medium and the suspension was spread on the surface of a combined nitrogen- and carbohydrate-free medium (Gyurján et al., 1984b) solidified with 2% agar. Growing green colonies were transferred to fresh medium in petri dishes monthly. Controls were pure algal and bacterial cell masses placed on the same medium. Settled associative cultures were examined at least once a year with the methods described below. Controls of these tests were axenic algal and bacterial cultures grown in their own media.

Measurement of chlorophyll content

Chlorophyll content of algal and symbiotic cultures was extracted in 80% (v/v) acetone and estimated from the absorbance data measured at 642 and 661 nm according to Avron (1960).

Measurement of photosynthetic O_2 evolution

An oxygen sensitive RA-9503 type combined Ag/Pt electrode was used for measuring O_2 evolution in light. Cell density was approximately 10^6 cells × cm^{-3} while temperature and illumination were 25°C and 2×10^4 lux, respectively.

Fluorescence induction measurements

Fluorescence induction curves (fluorescence rise) were measured by a newly developed apparatus (Chlorophyll fluorometer, Micro and Mikrolabor GMK, Szeged) (Szigeti et al., 1989). The characteristic parameters derived from the curves are means of 3 independent determinations.

Electron microscopy

Harvested cells were fixed in 1% glutaraldehyde (2 mM K-Na phosphate buffer, pH 7.2) for 2 hr and post-fixed in 1% OsO_4 using the same buffer for 2 hr. The samples were dehydrated in ethanol series and propylene oxide, then embedded in Durcupan (Fluka). Thin sections were made with a Porter-Blum MT-1 ultramicrotome, stained with uranyl acetate and lead citrate and then examined with Tesla BS-500 electron microscope.

For quantitative stereology cells harvested from a petri dish were fixed, embedded and sectioned as described above. In each analysis n=90 sections were randomly selected and electron micrographs (4000×) were made. The distribution of components was analyzed on enlarged prints (13,200×) using a square lattice grid with 0.5 cm distance between the line intersections. Intersections over the algae, bacteria and background were counted on an area of 50 × 60 (1800 altogether) intersections. From these data the mean values ($\bar{x}$) and their standard deviations ($s_{\bar{x}}$) were determined. It was possible to calculate the volume ratios directly because according to the Delesse rule in case of spherical structures with a random distribution the area and volume ratios are the same. The volume fractions were also determined which represent the proportions of different components (algal, bacterial and background) in the unit/whole volume.

Scanning electron microscopy

The cells were spread over a small aluminium foil in thin layer and fixed in 2.5% glutaraldehyde at room temperature for 1 hr and washed in 0.1 M cacodylate buffer, pH 7.4. Then the specimens were washed three times in the buffer at room temperature, dehydrated in ethanol series and amylacetate, and dried using CO_2 in a Du Pont critical point dryer (CT 06470). All specimens were mounted on stubs with a Dotite silver adhesive and coated with gold. Examinations were made with a Jeol 50/A scanning electron microscope at 15 kV.

3. Results

Cultures of *Ch. reinhardtii* on a nitrogen containing medium have a green colour. Upon nitrogen deprivation the colonies even in light turn yellow due to a drastic decrease in the chlorophyll content. In the alga-bacterium mixed cultures grown on nitrogen- and carbohydrate-free medium, green colonies appeared within some weeks. The first association was set up five years ago and provided a stable and functioning symbiosis which retained the green colour up to now. The partners probably divide and grow in balance because neither of them overgrows the other. Unsuccessful symbioses or control cultures do not grow in the medium used and both the algae and bacteria die soon.

Experimental results show that the partners retain their normal physiological state even when light is the sole energy-source. The photosynthetic activity in symbiosis shows a lower level compared to the control algal cultures (Table 1). The decrease in oxygen evolution is between 30 and 40%. The vitality indices calculated from chlorophyll-a fluorescence data decrease approximately by 50%. The acetylene reducing capacity of symbiotic bacteria was on an average 84% that of the free azotobacters (Nghia, 1986).

Data concerning enzyme activities show an integration on the metabolic level because in the case of diaphorase and isocitrate dehydrogenase, new enzyme patterns are formed in the associative culture (Nghia et al., 1986). An elevated algal glutamine synthetase activity was also observed in the association.

On scanning and transmission electron micrographs, the partners can be seen in close contact with each other and the bacteria are often located on the surface of the algal cells (Fig. 1). On transmission electron micrographs, bacteria are seen in great number in the intercellular spaces of the algal colonies (Fig. 2). From the electron micrographs it was possible to calculate the relative volume ratios of the partners (Table 2) which have not changed significantly during the five years.

Table 1. Photosynthetic activity of *Chlamydomonas-Azotobacter* symbiosis characterised by F_v (vitality index calculated from chlorophyll-a fluorescence measurements) and O_2 evolution

Objects	Photosynthetic activity			
	F_v	%	O_2 evolution	%
Wild type *Ch. reinhardtii*	61	100	4.9	100
Ch. — *Azotobacter* symbiosis 1	26	42	3.1	63
Ch. — *Azotobacter* symbiosis 2	48	69	3.0	60

Table 2. Quantitative analysis of alga-bacterium symbiosis using electron micrographs (n=90, number of counted line intersections=1800)

mean line intersections $\bar{x} \pm s_{\bar{x}}$			mean area or volume ratios			volume fractions		
A	B	C	A/B	A/C	B/C	A/V	B/V	C/V
694.4±32.7	17.2±1.7	1114.7±30.3	40.5	0.62	0.02	0.39	0.01	0.60

A = alga, B = bacterium, C = background, V = total volume

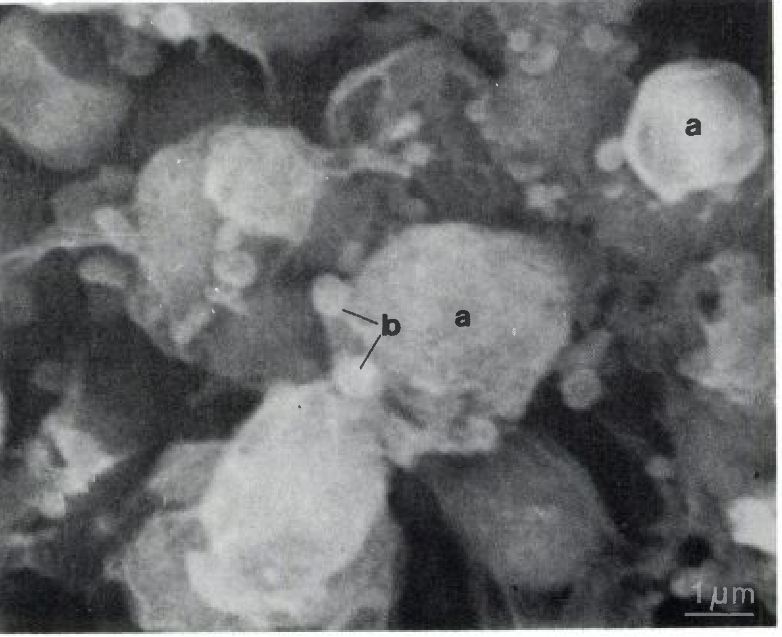

Figure 1. Scanning electron micrograph of alga-bacterium associative culture. a = alga, b = bacterium. (magn. 10,000×).

Frequent algal divisions (Fig. 3) indicate that algal cells can obtain enough nitrogen from bacteria for their growth. The bacteria are also dividing, suggesting that associative algal cells can support sufficient growth of azotobacters.

In some cases, bacteria are also seen in the cytoplasm of the algal cells. Greater magnifications show that these solitary bacteria are in membrane-bound vesicles (Fig. 4).

4. Discussion

We have established a stable association between originally non-symbiotic nitrogen-fixing bacteria and photosynthetic unicellular green algae simply by mixing the organisms on a selective medium. The experimental results show that the *Azotobacter-Chlamydomonas* system grows and is viable for a long period of time. The partners divide and grow, can be maintained in balance and their metabolism is not damaged but rather adapts to the mutual requirements.

The metabolic activity was characterized by photosynthetic O_2-evolution and vitality index. From the O_2-evolution data it is obvious that in symbiosis

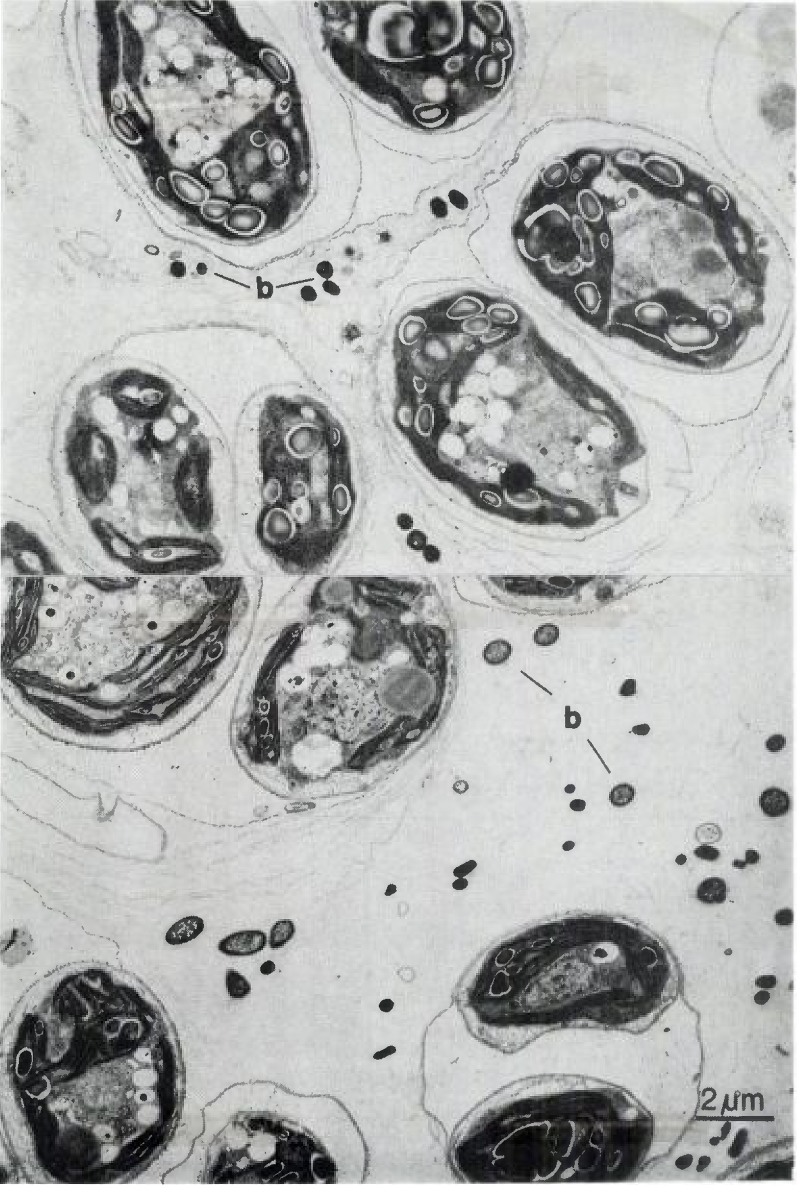

Figure 2. Bacteria in the intercellular spaces of alga colonies. b = bacterium. (magn. 5060×).

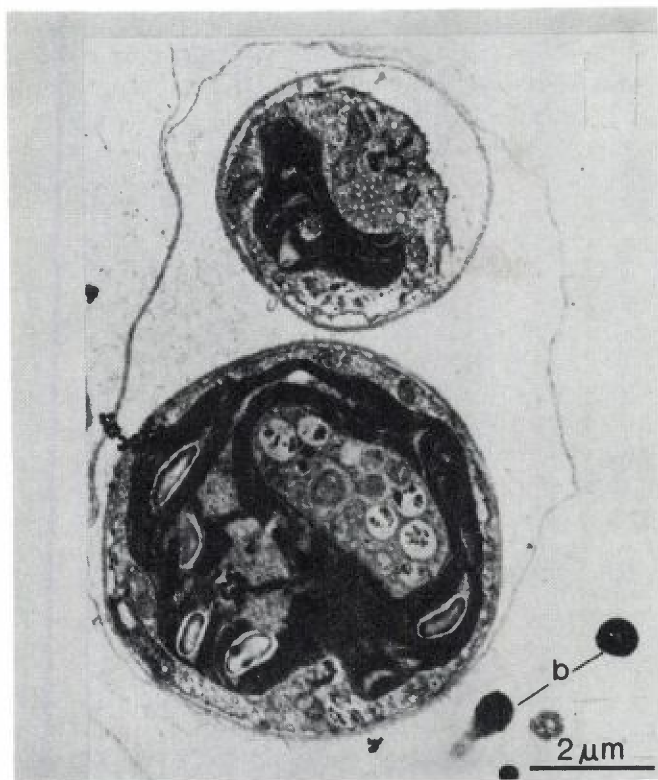

Figure 3. Algal cells immediately after division. (magn. 9200×)

the rate of photosynthesis exceeds the respiratory rate and for this reason more energy is formed than consumed. The decrease of vitality index does not refer to a damage of the photosynthetic pigment system. The acetylene assays showed that azotobacters are able to fix molecular nitrogen in the partnership. These observations suggest that the partners retain their normal physiological state in the associative symbiosis.

The experimental fact that our symbiosis is capable of autotrophic growth suggests that the algae provide carbon sources to their symbionts and the bacteria fixed nitrogen to their hosts. However, the exchange of nutrients has only indirect proofs. Diazotrophic *Azotobacter* species excrete ammonia and other compounds such as amino acid, vitamins and hormones into their media (Newton et al., 1953; Kleiner and Kleinschmidt, 1976; Kuhla et al., 1985; Gonzalez-Lopez et al., 1983; Karube et al., 1981). On the other hand, photosynthetically active *Ch. reinhardtii* cells release proteins, polysaccharides and glycolate into the medium (Kroes, 1972; Tolbert, 1979; Vela and Guerra, 1966). Nghia (1986) proved that *Azotobacter vinelandii* can utilise glycolate

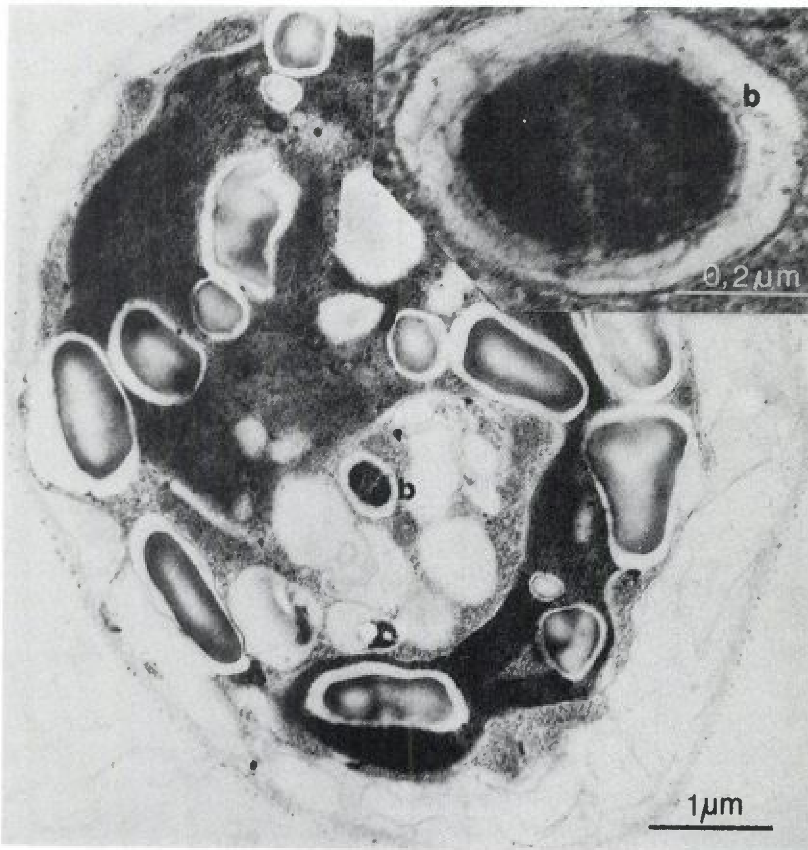

Figure 4. Symbiont bacterium in membrane-bound vesicle inside the algal cytoplasm. b = bacterium. (magn. 18,400×, 101,200×)

as a carbon source although the rate of growth decreases by 70% compared with controls grown on glucose containing medium. According to his results it can be assumed that ammonia derived from nitrogen fixation of azotobacters is transferred to algal cells and assimilated by glutamine synthetase. Because of the lack of direct evidence for metabolite exchange, studies utilizing radiolabeled compounds would be useful in the future.

On electron micrographs, many bacteria are near the surface of the algal cells and in the intercellular spaces. Consequently our system can be described as an ectocytobiosis. The steady ratio of partners may refer to their metabolic cooperation. Morphologically, the bacteria and algal cells divide as in pure cultures.

On some pictures the bacteria could be localized in membrane-bound vesicles

inside the cytoplasm of the algal cells. This may be related to local nitrogen deficiencies appearing during the culture. It is known (Sager and Granick, 1954) that nitrogen deprivation induces gametogenesis in *Ch. reinhardtii* and wall-less opposite mating type gametes form from the vegetative cells. The gametes which have shed their walls can fuse with the help of a fertilization tubule. In mixed cultures the bacteria can be randomly taken up into zygotes during the fusion process. This explanation for the formation of endocytobiotic alga-bacterium symbioses was proved experimentally (Nghia et al., 1986).

We can conclude that in the *Azotobacter-Chlamydomonas* association there is a stable and coordinated relation between the partners. The described mechanism provides at least one example whereby endocytobionts can be harbored by cell-walled cells which are generally not regarded as hosts. Thus, nitrogen deficiency besides the maintenance of autotrophic state induces the formation of endocytobiosis which could initiate some kind of organellar development. Our system provides a model for the establishment and analysis of cellular symbiosis and by the modification or change of partners it may be possible to obtain partnerships of greater practical significance.

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